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The major cause of cancer deaths can be attributed to metastasis. Our goal is to identify metastasis-controlling genes for human breast cancer. This research is based upon our finding that microcell-mediated transfer of chromosome 11 into MDA-MB-435 results in nearly complete suppression of metastasis without suppressing tumorigenicity.

The key findings during this reporting period were: (1) demonstration that *KiSS-1*, a melanoma metastasis-suppressor gene, caused significant suppression of metastatic potential of MDA-MB-435 human breast carcinoma cells; (2) demonstration that *Kai-1* mRNA expression levels correlated with metastatic potential of a panel of human breast carcinoma cell lines; and (3) showing that transfection of *Kai-1* resulted in partial suppression of metastasis.

Preliminary results include: (1) Using differential display, we also identified several differentially expressed genes in neo11/435 hybrids compared to controls. Initial findings were complicated by overabundance of retroviral sequences (presumably resulting from method of 11 donor derivation). However, some cDNAs fragments were apparently novel. Further characterization is underway. (2) To prepare for transfer into MDA-MB-435, we identified and characterized a panel of chromosome 11 with deleted regions. Some hybrids have been made and are being characterized.

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Danny R. Hall — 8 July 1997

PI - Signature — Date

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INTRODUCTION

The most dangerous attribute of cancer cells is metastasis. Our objective is to determine the molecular mechanisms responsible for controlling breast cancer spread. The timing and location of nonrandom karyotypic abnormalities has provided clues regarding the genes involved in breast carcinoma progression. In breast cancer, structural changes frequently involve chromosomes 1, 8, 11, 13, 16 and 17. Chromosomes 8, 13 and 17 changes generally occur early in progression; whereas, deletions and rearrangements of chromosomes 1, 6, 11 and 16 often occur later (Walker et al., 1997). As a corollary, one would hypothesize that genes relevant to breast cancer progression toward metastasis are encoded on the latter chromosomes. To test this hypothesis, we introduced an intact, normal human chromosome 11 into the metastatic human breast carcinoma cell line, MDA-MB-435 using microcell-mediated chromosome transfer (MMCT). We showed that metastasis was suppressed by 95%, but tumorigenicity was unaffected (Phillips et al., 1996). This finding suggested the presence of at least one human breast carcinoma metastasis-suppressor gene on chromosome 11. ***Please note***: We define a metastasis-suppressor gene as blocking tumor spread. A *tumor* suppressor gene would suppress tumor growth and, by inference, metastasis as well.

The goal of DAMD-17-1-96-6152 is to extend our findings in order to map the gene(s) on chromosome 11 responsible for metastasis suppression. In addition, we want to test whether similar metastasis suppression occurs if chromosome 11 is introduced into other metastatic human breast carcinoma cell lines. To alleviate repetition in various sections below, I will provide a brief background highlighting the key material necessary to understand our approaches and strategies.

The metastasis problem

Metastasis is the ultimate step of tumor progression toward autonomy from the host (i.e., less-dependent and less-responsive to normal growth regulatory signals) resulting from accumulated genetic changes (Welch and Tomasovic, 1985; Miller and Heppner, 1990; Nicolson, 1993; Welch, 1997). As a result of selective pressures, a subset of late-stage cancer cells are no longer confined to their tissue of origin for growth. In order to successfully colonize a distant organ, metastatic cells must survive transport through the body, interact with a variety of host cells and successfully penetrate numerous barriers. If a cell cannot complete every step, it is nonmetastatic. It is clear, then, that the metastatic cascade involves numerous genes (Wright et al., 1990; Nicolson, 1991; Welch and Tomasovic, 1985; Miller and Heppner, 1990). There is evidence for two classes of metastasis-associated genes — (i) genes that drive metastasis formation, and (ii) genes that inhibit metastasis (Wright et al., 1990; Nicolson, 1991; Welch and Tomasovic, 1985; Miller and Heppner, 1990; Liotta, 1992; Dickson and Lippman, 1992; Nicolson, 1993; Steeg, 1989; Dear and Kefford, 1990; Sobel, 1990; Liotta and Stetler-Stevenson, 1991; Steeg et al., 1993; Welch et al., 1994). However, the identities of most of these genes remain unknown. Correspondingly, it is unknown how these genes are regulated in normal and/or cancer cells. Nonetheless, it is well recognized that the probability for long-term survival is extremely low if metastases develop.

Genetics of cancer metastasis

Several metastasis-associated genes have been identified, yet only two have been shown to suppress metastasis when introduced into human cells — Nm23-H1 and KiSS-1. Nm23 was originally discovered by Dr. Patricia Steeg at the National Cancer Institute using subtraction hybridization of variants of the K1735 murine melanoma (Steeg et al., 1988). Her laboratory subsequently cloned the human homolog, NME1 or Nm23-H1, and showed that transfection into the metastatic human breast carcinoma cell line MDA-MB-435 caused suppression of metastasis in a nude mouse assay by approximately 50% (Leone et al., 1993). Our laboratory, in collaboration with hers, recently showed that NME1 also suppresses metastasis in some, but not all, human melanoma cell lines (Miele et al., 1997a).

KiSS-1 was identified by our laboratory in human melanoma cells and has been shown to suppress melanoma metastasis by a minimum of 50% (Lee et al., 1996). KiSS-1 is a novel gene but its mechanism

of action is not yet known. However, its location on chromosome 1q32-q41 was surprising since gene expression was induced by addition of human chromosome 6 in MMCT experiments analogous to those described above (Lee et al., 1996). In five metastatic human melanoma cell lines, transfection results in ≥50% suppression of the metastatic phenotype (Lee and Welch, 1997). Based upon its metastasis suppressor effects and the chromosomal location, we hypothesized that KiSS-1 might also function in breast cancer. Data presented below will show that KiSS-1 also suppresses metastasis when transfected into MDA-MB-435.

The Kai-1 gene was recently isolated by Dong and colleagues in a rat prostatic carcinoma model (Dong et al., 1995). The human homolog was cloned and maps to chromosome 11p11.2. Since this gene maps to chromosome 11, we suspected that it might be responsible for metastasis suppression in the chromosome 11/MDA-MB-435 hybrids. Initial RNA blotting experiments showed higher levels of Kai-1 in neo11/ MDA-MB-435 (Phillips et al., 1996), further supporting our suspicions. Transfection experiments were initiated to test this hypothesis. Data presented below provide a mixed conclusion regarding the role of Kai-1 in controlling breast cancer metastasis.

BODY
-EXPERIMENTAL METHODS-

Rationale (Global)

Positional cloning has been used to identify a number of tumor-suppressor genes (e.g., WT1, Rb, FHIT) and genes for mutations predispose cancer susceptibility (e.g, NF1, APC) (Stanbridge, 1990). As mapping nears completion, detection of mutations among cancer families confirms a particular gene's role as a tumor suppressor. Since mutations are relatively rare, equally strong evidence for a role in cancer etiology is required. Thus positional cloning is a reasonable approach if strong, well-characterized pedigrees are available. However, determining roles for genes in sporadic tumors or progression-associated genes (e.g., metastasis-controlling), is more difficult because of tumor heterogeneity, genetic instability and the huge number of experiments necessary to prove causality. Simply, the statistical likelihood for finding a particular gene over a background of genetic instability is difficult. This is made all the more apparent in late-stage cancers where accumulated genetic changes are nearly maximal. Thus, alternative approaches are required. MMCT has been extremely useful in providing functional evidence for tumor suppressor genes when other approaches have failed (Anderson and Stanbridge, 1993; Hunt, 1996). MMCT data have been followed by successful mapping of the genes (Chen et al., 1994; Saxon et al., 1986; Loh et al., 1992; Trent et al., 1990; Chen et al., 1994; Church et al., 1993; Gioeli et al., 1997; Koi et al., 1997; Kuramochi et al., 1997; Reiss et al., 1997; England et al., 1996; Coleman et al., 1995; Ewing et al., 1995; Ohmura et al., 1995; Theile et al., 1995). Some have utilized a modification of MMCT in which the donor chromosome has been irradiated to produce deletions (Dowdy et al., 1990; Koi et al., 1993; Murakami et al., 1995; Gioeli et al., 1997; Plummer et al., 1997; Coleman et al., 1995). This modification is based upon a loss of function (i.e., failure to suppress) associated with the deletion.

The strategies we proposed for identifying metastasis-controlling genes in human breast cancer were based upon those we successfully used to identify novel metastasis-suppressor genes in human melanoma (Welch et al., 1994; Jiang et al., 1995; Lee and Welch, 1997; Lee et al., 1996; Miele et al., 1997b). Basically, two concurrent approaches were outlined. First, progressively smaller fragments of neo-tagged human chromosome 11 were to be introduced into MDA-MB-435 by MMCT. By evaluating regions of overlap for chromosomal fragments present/absent in suppressed/non-suppressed hybrids, the location of the putative metastasis-suppressor gene(s) would be defined. The second approach was to use differential display (Liang and Pardee, 1992; Liang et al., 1993) and subtractive hybridization (Ausubel et al., 1990; Hutchins et al., 1991). Once candidate genes were identified, transfections and testing for metastasis in appropriate animal models would confirm that a *bona fide* metastasis-suppressor gene had been cloned.

The second major objective of DAMD-17-1-96-6152 was to demonstrate the introduction of chromosome 11 into another metastatic human breast carcinoma also suppresses metastasis.

Each section below summarizes results from related series of experiments performed in relation to the Specific Aims proposed. Each section is outlined based upon publications detailing the work. Where the results have been published, only a summary is presented. Raw data is included for unpublished results.

Section 1: **Suppression of MDA-MB-435 breast carcinoma cell metastasis following the introduction of human chromosome 11 [*Cancer Research* (1996) 56:1222-1227]**

Summary of major findings

MMCT was used to introduce an additional copy of a normal human chromosome 11 into MDA-MB-435. Hybrids (neo11/MDA-MB-435) were still tumorigenic, but were suppressed for their ability to form metastases following injection into the mammary fat pads of athymic nude mice. *In vivo* growth rates were unaffected or, in many cases, faster than the parent. Therefore, metastasis suppression could not be explained by slow growth. Tumors growing at the site of mammary fat pad injection were still locally invasive in H&E stained sections, suggesting that the step(s) of metastasis affected by addition of chromosome 11 are subsequent to invasion and detachment from the primary tumor. All cultured cell lines and DNA isolated from primary tumor tissue retained the added 11.

Controls included two single cell clones (designated MDA-MB-435.1 (435.1) and MDA-MB-435.7 (435.7)) that were isolated by limiting dilution and tested for metastasis *in vivo*. The neo11/MDA-MB-435 hybrids were significantly less metastatic than both clones. The "irrelevant" chromosome control was done with chromosome 6 using 435.7. The rationale was that starting with a recently derived clone should minimize the impact of heterogeneity and phenotypic drift (Welch et al., 1983) in the parent populations. While 435.7 had only a single copy of chromosome 6, it was poorly metastatic. Despite this complication, the results suggested that chromosome 6 did not suppress MDA-MB-435 breast carcinoma metastasis.

Metastasis-suppressed neo11/MDA-MB-435 hybrid cell clones express significantly more Kai-1 mRNA. Dr. J. Carl Barrett and colleagues recently described a new metastasis-suppressor in human and rodent prostatic carcinomas, Kai-1, (Dong et al., 1995). Since Kai-1 is encoded at 11p11.2, and since this gene falls near the region of previously described deletion in human breast cancer, we examined neo11/MDA-MB-435 cells for upregulation of this gene. Significantly more mRNA was expressed. Nm23-H1 expression was not changed in neo11/MDA-MB-435 hybrids.

Recommendations for follow-up experiments based upon these results

1. The metastasis suppression result requires validation in another, independently-derived human breast carcinoma cell line. (See Section 2)

2. Additional single cell clones should be evaluated for two reasons. First, the inter-experimental variability of metastatic potential in the parental cells is high, making statistical evaluation difficult. Having a single cell clone with reproducibly high metastatic potential would provide a greater differential for observing suppression. Second, it is clear that MDA-MB-435 is more heterogeneous than is represented by 435.1 and 435.7. (See Section 3)

3. Since metastasis suppression was not 100%, establishment of revertants would be most useful only after more highly metastatic populations were obtained.

4. Additional "irrelevant" chromosome transfers are advisable.

5. Kai-1 could be the gene responsible for metastasis suppression. Therefore, transfection experiments should be done. (See Sections 4 and 5)

6. While Nm23 is known to suppress MDA-MB-435 metastasis, it does not appear to be involved in the suppression following introduction of chromosome 11. Therefore, further studies with Nm23 are not advised.

7. MMCT using smaller pieces of chromosome 11 are required. (See Section 6)

Section 2: **Introduction of chromosome 11 into MDA-MB-231 [Unpublished]**

Rationale

The objective of this set of experiments is to determine whether introduction of chromosome 11 into another human breast carcinoma causes metastasis suppression.

Summary of major findings

Although most human breast carcinoma cell lines were derived from metastatic lesions or pleural effusions, most do not metastasize in experimental animal models (Welch, 1997). The MDA-MB-231 has been reported in the literature to be metastatic (Price et al., 1990). Therefore, we obtained these cells from Dr. Garth Nicolson (Institute for Molecular Medicine, Irvine, CA). Before initiating labor-intensive experiments involving MMCT, we decided that metastatic potential should be verified in our hands beforehand. When cells (up to 1×10^7) were injected into the mammary fat pads of athymic nude mice, no tumors formed. Upon checking with Dr. Nicolson, the cells had apparently been contaminated with *Mycoplasma* and rescued. We concluded that a nontumorigenic subpopulation had inadvertently been isolated. So, we requested another isolate from Dr. Robert Gillies (University of Arizona Cancer Center, Tucson, AZ). When injected into athymic nude mice, only 18/25 animals developed tumors. Of those mice, 1/18 had a ipsilateral axillary lymph node metastasis.

Recommendations for follow-up experiments based upon these results

1. The variants of MDA-MB-231 that we have thus far obtained are clearly nonmetastatic in athymic nude mice. Dr. Gillies reports to us that he now routinely uses SCID mice rather than nude mice. We are planning to measure metastasis in SCID mice in mid-summer 1997. Perhaps this will need to be a standard for future experiments.

2. We have also been in contact with Dr. Janet Price (U.T.-M.D. Anderson Cancer Center, Houston, TX) who may have recently isolated a new metastatic human breast carcinoma cell line. She has agreed to provide this variant upon completion of her initial characterization.

3. In addition, we have contacted Dr. Steven Ethier (University of Michigan) who has an USAMRDC Infrastructure Grant. Dr. Ethier has provided us with two breast carcinoma cells that exhibit *in vitro* properties which he believes may suggest that they may be metastatic *in vivo*. We intend to evaluate these cells *in vivo* as potentially useful for future studies.

Section 3: **Isolation of MDA-MB-435 single cell clones**

Rationale

Since tumors are heterogeneous mixtures of cells, an important control for MMCT is characterization of single cell clones. The purpose of this set of experiments was to isolate single cell clones. Also, we were hoping to identify more highly metastatic variants for use in subsequent experiments. By starting with a more highly metastatic variant, we reasoned that our metastasis suppressor assay would be more sensitive. This could be important once candidate genes are being evaluated, especially if suppression is not complete.

Summary of major findings

Limiting dilution cloning was used to isolate single cell clones from the parental MDA-MB-435 population. Similar experiments were performed with pSV2neo-transfected and pcDNA3neo-transfected MDA-MB-435 cells. The neo-transfectant clones were isolated in order to control for MMCT experiments and as part of transfection experiments (See below).

Recommendations for follow-up experiments based upon these results

1. It has been frustrating that the metastatic potential of single cell clones has been so variable (inter-experimental). Most clones were tested at least twice during the past year. In general, trends were the same; however, there is still more variability than desired. Use of the mixed population, while not ideal, still represents the best option at this point.

2. We will attempt to select, in a manner analogous to Fidler (Fidler, 1973), increasingly metastatic subpopulations from MDA-MB-435. This will provide a more highly metastatic variant. Thus, we will have a higher baseline from which to observe suppression.

Table 1. Summary of MDA-MB-435 metastasis assays completed under grant DAMD-17-96-1-6152 from July 1, 1996-June 30, 1997

Cell Line	No. Lung Metastases per mouse (Mean ± S.E.M.)	Median (range)
MDA-MB-435	23 ± 5	4 (0, >250)
Single cell clones		
MDA-MB-435.1	24 ± 10	13 (3, >250)
MDA-MB-435.7	11 ± 4	7.5 (0, 75)
MDA-MB-435.A	47 ± 20	22.5 (0, 167)
MDA-MB-435.B	1.8 ± 0.7	1 (0, 8)
MDA-MB-435.D	8 ± 3	5 (0, 46)
MDA-MB-435.F	16 ± 4	15 (1, 62)
MDA-MB-435.H	17 ± 8	9 (0, 130)
MDA-MB-435.J	11 ± 1	10.5 (0, 20)
Neo-transfected MDA-MB-435		
MDA-MB-435 neo.1 (pcDNA3)	20 ± 8	10.5 (3, 71)
MDA-MB-435 neo.2 (pcDNA3)	9 ± 4	4.5 (0, 35)
MDA-MB-435 neo.A8 (pSV2neo)	9 ± 4	4.5 (0, 35)
MDA-MB-435 neo.B8 (pSV2neo)	8 ± 5	2 (0, 37)
MDA-MB-435 neo.C8 (pSV2neo)	1 ± 0.3	1 (0, 2)
MDA-MB-435 neo.D8 (pSV2neo)	2 ± 0.4	1.5 (1, 4)
MDA-MB-435 neo.E8 (pSV2neo)	4 ± 1	0.5 (0, 9)
Transfectants — KiSS-1		
MDA-MB-435 KiSS-1 mix	1 ± 1	0.5 (0, 6)
MDA-MB-435 KiSS-1.1	5 ± 2	2.5 (0, 21)
MDA-MB-435 KiSS-1.2	2 ± 1	1.5 (0, 7)
MDA-MB-435 KiSS-1.3	1 ± 0.4	0.5 (0, 2)
MDA-MB-435 KiSS-1.7	3 ± 1	3 (0, 10)
Transfectants — KiSS-1		
MDA-MB-435 Kai-1 pick-1	18 ± 10	1 (0, >200)
MDA-MB-435 Kai-1 pick-2	15 ± 12	1 (0, >200)
MDA-MB-435 Kai-1 pick-3	11 ± 4	6 (0, 67)

Data presented are cumulative for 1-9 experiments per cell line. Cells (10^6) were injected into the sub-axillary mammary fat pads of 4- to 6-week-old female athymic nude mice. For most studies, primary tumors were removed when mean tumor diameter reached 1.0-1.3 cm. Metastases were quantified 4 wk later. For neo-transfectants, vector used is shown in parentheses

Section 4: **Reduction of metastasis in the MDA-MB-435 model system**

correlates with increased expression of Kai-1 protein [*Molecular Carcinogenesis* (1997) accepted pending minor revisions]

Summary of major findings

Since introduction of chromosome 11 suppressed metastasis of MDA-MB-435 and a putative metastasis-suppressor gene, Kai-1, maps to chromosome 11, we tested whether Kai-1 was responsible for metastasis suppression. Two lines of investigation were carried out in collaboration with Drs. Bernard Weissman (Lineberger Cancer Center, Chapel Hill, NC) and Lisa Wei (Lombardi Cancer Center, Georgetown University, Washington, D.C.). With Dr. Weissman, we evaluated whether transfection of Kai-1 would suppress MDA-MB-435 metastasis. The text of that manuscript is printed below. The data collected with Dr. Wei is presented in Section 5.

Abstract

We have previously shown that human chromosome 11 contains a functional metastasis-suppressor gene for breast cancer by microcell-mediated transfer of a normal chromosome 11 into the highly metastatic MDA-MB-435 human breast carcinoma cell line. A known metastasis-suppressor gene, Kai-l, and a related family member, TAPA-l, have been mapped to chromosome 11p11.2 and 11p15.5, respectively. To determine if these genes were responsible for the metastasis suppression seen in our breast carcinoma MMCT hybrids, we examined their expression by Western blot analysis. Although TAPA-1 expression did not correlate with metastasis suppression, Kai-1 production was greatly diminished in the MDA-MB-435 cells and increased in the metastasis-suppressed chromosome 11 MMCT hybrids. Chromosome 6 MMCT hybrid controls, which remained metastatic, did not have increased levels of the Kai-1 protein. Transfection of full-length Kai-1 cDNA into MDA-MB-435 cells did not result in a significant decrease in the *in vivo* incidence of lung metastases. However, Western blot analysis showed that the primary tumors and the metastatic lesions of the transfectants had decreased levels of Kai-1 protein compared to the inoculated cells. Furthermore, several of the transfectant clones expressed heavily modified Kai-1 protein compared to that of the microcell hybrids. Our data indicate that protein modification may affect the normal function of Kai-1 *in vivo* and/or a threshold level of Kai-1 protein expression may be necessary for suppression of the metastatic phenotype.

Introduction

Cytogenetic analysis of metastatic breast tumors has shown that chromosome 11 rearrangements often appear as late events in breast cancer (Trent et al., 1993), suggesting the presence of a metastasis-suppressor gene. Yet, it has become clear that changes on chromosome 11 may also contribute to tumor initiation for breast cancer, since the recently cloned aim gene at 11q22-23, when mutated, predisposes even heterozygotes to breast cancer (Swift et al., 1991; Savitsky et al., 1995). Other tumor-suppressor genes for different tumor types have been identified on chromosome 11 (Call et al., 1990; Gessler et al., 1990; Hao et al., 1993), and several more loci are under intense investigation (Reid et al., 1996; Ali et al., 1987; Hampton et al., 1994). Additionally, the α2-integrin gene, which maps to chromosome 11 has been implicated in malignancy (Zutter et al., 1995). Therefore, to fully comprehend the mechanisms involved in tumor progression, it may be critical to identify changes in gene expression on chromosome 11 for all stages of breast carcinoma, Wilms' tumor, and other cancer types.

Previous work in our laboratory has shown that the introduction of human chromosome 11 into the highly metastatic MDA-MB-435 breast carcinoma cell line significantly reduces the metastatic potential of the hybrid clones (Phillips et al., 1996). Metastasis suppression was not observed with the control chromosome 6 microcell hybrids. This experimental evidence indicated that chromosome 11 harbors a metastasis-suppressor gene for human breast cancer.

To date, only a few metastasis-suppressor genes have been isolated from the human genome. The Kai-1 gene, also known as CD82 (C33 antigen), maps to 11p11.2 and codes for a glycoprotein in the transmembrane 4 superfamily (TM4SF) (12-14). Since it is operative in suppressing prostate cancer

metastatic ability (Dong et al., 1995), Kai-1 is also an attractive candidate for a breast cancer metastasis-suppressor gene on chromosome 11. In addition, the gene for another TM4SF member, TAPA-1 (CD81), also maps to chromosome 11 on band pl5.5 (Takahashi et al., 1990). Although TAPA-1 has not been implicated in cancer suppression, it has been shown to induce cell-cell adhesion (Takahashi et al., 1990; Virtaneva et al., 1994; Wright and Tomlinson, 1994), a property often altered in metastatic cells (Trent et al., 1993). The functions of these TM4SF surface proteins are largely unknown. They associate with cell surface receptors and are components of signaling complexes (Bradbury et al., 1992) and may be important for maintenance of cell integrity, proliferation, and adhesion (Nojima et al., 1993). Therefore, alterations in expression levels of the Kai-1 and/or TAPA-1 proteins could result in acquisition of invasive or metastatic ability.

We wanted to determine whether either Kai-1 or TAPA-1 might control human breast metastatic ability. By Western blot analysis, the chromosome 11 hybrids had increased levels of Kai-1 protein compared to the MDA-MB-435 parental cells. TAPA-1 protein levels were unchanged between the parent cell line and all microcell hybrids. To directly test the role of the Kai-1 gene in our system, we transfected Kai-1 cDNA into both a mass population of MDA-MB-435 and a well characterized subclone. No significant difference in the incidence of metastasis was observed between the transfectant clones and the MDA-MB-435 parental cells. However, analysis of the metastatic lesions and primary tumors by Western blotting revealed that Kai-1 levels had been reduced in these tissues. These data indicate that progression to metastasis in breast cancer may occur by a mechanism which down regulates Kai- 1 protein production.

Materials And Methods

Cell Lines. Chromosome 11 (neo11/435) and chromosome 6 (neo6/435.7) microcell hybrids were generated as previously described by introducing single human chromosomes tagged with the neo-resistance gene into the highly metastatic MDA-MB-435 human breast carcinoma cell line (Phillips et al., 1996). Chromosome 11 hybrids (neo11/ 435.A3, B1, D1, and E1) were derived from fusions using the mass population of MDA-MB-435 cells. Chromosome 6 hybrids (neo6/435.7.Al, E2, and G1) were derived from fusions using a subclone of the mass population. Transfected cDNA clones, designated Kai 1/435.Picks 1, 2, 3, and 4 (note: these are <u>not</u> necessarily clonal), were generated after lipofectin-mediated transfection of the MDA-MB-435 cells with supercoiled 8.2 kb pCMV-kail plasmid. cDNA for Kai-1 was obtained as previously described (Dong et al., 1995) by human-specific Alu element-mediated PCR on DNA unique to prostate clones suppressed for metastasis. Cloned fragments were used to screen a cDNA library, resulting in a clone for the Kai-1 gene. To construct the transfection plasmid, the 1.6 kb Kai-1 Sal I insert was ligated into the Xho I site of the 6.6 kb pCMV-Neo-Xho I expression vector. Transfectants were also made from MDA-MB-435.subclone 1 cells, and these were designated Kail/435.subl.Pick-1, subl.Pick3, and subl.Pick4. The expression vector alone was used to generate neo-transfected control clones, designated neo/435.A8, B8, C8, D8, and E8. MDA-MB-436 cells are a highly invasive (Thompson et al., 1992) and non-metastatic breast carcinoma which were purchased from the ATCC (Rockville, MD). 184 cells are immortal human mammary epithelial cells (Stampfer, 1985). Protein from 184 cell lysate was kindly supplied by Dr. Bruce Lessey, University of North Carolina, Chapel Hill. All cell lines were maintained in RPMI, 10% FBS, and microcell hybrids and transfectant clones were additionally supplemented with 600 ug/ml G418.

Western Analysis: Cells were harvested from near confluent T-75 flasks and lysed according to previously described protocol (Imai et al., 1992) 12.5% SDS-PAGE gels contained 10-25 ug of protein sample per lane. Protein was transferred to membranes (Immobilon-P; Millipore) and incubated overnight (PBS, 0.1% Tween-20, 10% dry milk) before the addition of C33 (Kai-1) antibody. Detection of C33 antibody was done using standard ECL techniques (Amersham). The C33 (Imai et al., 1992) and TAPA-1 (Takahashi et al., 1990) monoclonal antibodies were kind gifts from Osamu Yoshie and Shoshana Levy, respectively.

PCR Analysis: The presence of the Kai-1 cDNA in the transfected cell lines was confirmed by PCR. Genomic DNA was amplified using previously described primers (Dong et al., 1995) for the cDNA insert, yielding an amplicon of approximately 1 kB. PCR solutions contained 400 ng genomic DNA from parental cells and transfectants using standard buffer components and 1.0 mM MgCl2. Reaction conditions (50 ul) consisted of 30 cycles at 94°C, 1 min; 62°C, 2 min.; and 72°C, 2 min. Amplicons were visualized in ethidium bromide-stained 2% agarose gels.

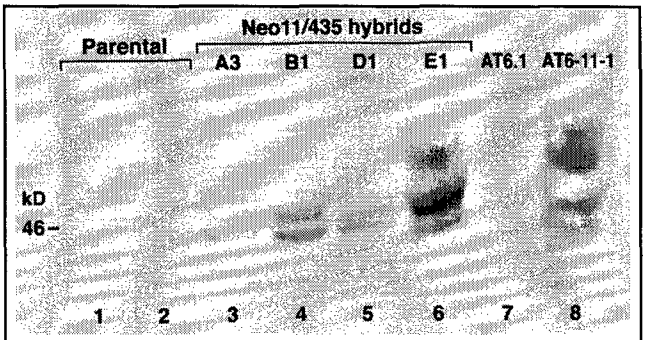


Figure 1: Western blot of Kai-1 protein levels of MDA-MB-435 and MDA-MB-435.1 parental cells (Lanes 1 and 2), neo11/435 hybrids (lanes 3-6) and negative (AT6.1) and positive (AT6-11-1) control cell lines. (AT6-11-1 is a metastasis-suppressed chromosome 11 MMCT hybrid of the highly metastatic AT6.1 prostatic carcinoma cell line.

Spontaneous Metastasis Assay: Cells (1 x 10^6) were injected into the subaxillary mammary fat pads of three to five-week-old female, non-ovarectomized, athymic mice (Harlan Sprague Dawley, Madison, WI). Animals were sacrificed about 3 months after inoculation when the tumor size was ca. 2.0 cm or when the mice were moribund. In some mice the primary tumors were surgically removed at about 60 days, but the animals were not sacrificed until 90 days post-inoculation, in order to allow existing lung metastases to enlarge. Visible lung metastases were counted in Bouin's-fixed tissues as previously described (Welch et al., 1983; Phillips et al., 1996).

Results

Characterization of Kai-1 Expression in Parent and Microcell Hybrid Lines By Western Blot. To determine the Kai-1 protein levels of the metastatic MDA-MB-435 breast cancer cells and the four non-metastatic chromosome 11 microcell hybrids, lysates were prepared from each cell line for Western blot analysis with C33 antibody. As shown in **Figure 1**, almost none of the protein is expressed in parental MDA-MB-435 and MDA-MB-435.Sub 1 cells (lanes 12), however, each metastasis-suppressed neat 1/435 hybrid (lanes 3-6) has an increased level of Kai-1 protein. One of the hybrids, neo11/435.E1, expresses amounts of Kai-1 similar to a prostate microcell hybrid line (AT6.1-11-1), also suppressed for metastasis (Dong et al., 1995). Interestingly, this same microcell hybrid grew more slowly in culture and appeared larger and less spindle-shaped than the other hybrids (data not shown). As was previously shown with immunoprecipitation experiments (Fukudome et al., 1992), the normal size of Kai-1 protein varies from 40-75 KDa with 1-3 major bands corresponding to the N-glycosylation state of the protein. Kai- 1 in the chromosome 11 microcell hybrids falls within this range (46-56 KDa) and has the normal 2-3-band pattern. One of the hybrids neo11/435.E1, expresses amounts of Kai-1 similar to a prostate MMCT hybrid cell line AT6.1-11-1), that is also suppressed for metastasis.

Figure 2 compares the amount of Kai- 1 protein in the parental cells with chromosome 6 hybrids, chromosome 11 hybrids, neomycin transfectant controls, and two non-metastatic breast carcinoma cell lines. The five lanes with Lysate from the least-metastatic cells have highest levels of the Kai- 1 protein. The immortalized, non-tumorigenic breast cell line, 184, has the highest level of Kai-1 protein expression, while Kai-1 protein in MDA-MB-436 is somewhat lower, approximately equivalent to the levels in the neat 1/435 hybrids. The Kai- 1 protein bands smaller than 46 KDa in the 184 cell Lysate resulted from protein degradation in the sample. Kai- 1 protein in the neo 11/435 hybrids has normal size range and pattern compared to both 184 and MDA-MB-436 cells. A Western blot of TAPA-1 showed no difference in the levels of protein among the MDA-MB-435 cells and two of its subclones (lanes 1-3), suppressed hybrids and non-suppressed hybrids. These data strongly indicate that Kai-1, and not TAPA-1, expression in the neo11/435 microcell hybrids correlates with the ability to suppress metastasis.

Transfection of the Kai-1 gene into the MDA-MB-435 Cell Line. To determine if the increase of Kai-1

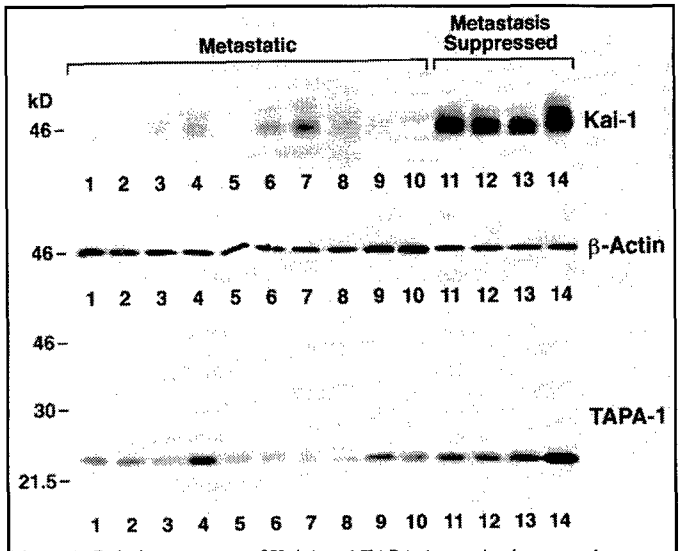


Figure 2: Relative amounts of Kai-1 and TAPA-1 proteins between the metastatic parental MDA-MB-435 parental population and two subclones (lanes 1-3); metastatic neo6/435.7. A1, .C1, .E2 and .G1 (lanes 4-7, respectively); neo-transfectant controls (lanes 8-10) and metastasis-suppressed neo11/435.A3, .B1, .D1 and .E1 (lanes 11-14, respectively). Lanes were loaded with 20 ug protein and probed with a 1:50 dilution of C33 antibody, then stripped and re-probed with a 1:1000 dilution of TAPA-1 antibody. Protein size is given in kDa to the left of each blot. The Western of beta actin was run on a separate blot made from denatured sample and was used to determine equal loading.

protein was responsible for the metastasis suppression seen in the chromosome 11 microcell hybrids, Kai-1 cDNA was transfected into the MDA-MB-435 cell line. Isolated colonies of presumed clonal origin, designated Kail/435.Pick 1, 2, 3, and 4, resulted from transfection of the Kai-1 cDNA into the mass population of MDA-MB-435 cells. Kai/435.sub 1.Picks 1, 3, and 4 clones resulted from transfection into the of MDA-MB-435.1 subclone. PCR was used to verify that the insert was successfully transfected into each clone. Five of the seven Kai-1 transfectants showed the expected 1 kb PCR product which the parental cells and the neo transfected control cell lines lacked. Kai/435.Pick 1 and Pick 3 did not amplify for the Kai-1 cDNA to the same extent as the other transfectant clones, and it is presumed that a full-length cDNA copy of the gene was not successfully introduced into these two transfectants. Therefore, Kai-1 protein expression from Kai/435.Pick 1, Pick 3, the parental cells, and the neo transfectants came from the endogenous genes.

Characterization of morphological differences in transfectants. To determine if variations in Kai-1 expression and glycosylation affect cell type, each of the microcell hybrids and transfectants were compared for cell size and shape in tissue culture. Because TM4SF proteins are cell surface molecules, the amount of Kai-1 expression and/or degree of glycosylation could presumably affect cell-cell interactions. **Figure 3** shows representative photographs of cell lines with liesser amounts of Kai-1 (left panels) beside cell lines with higher levels and more glycosylated levels of Kai-1 (right panels). The neo11/435.D1 cells are more compact and more spindle-shaped than the neo11/435.E1 cell line. Likewise, Kai-1 transfectants grew more closely in culture and the cells were smaller than parent.

To determine the level of Kai- 1 expression in the transfected clones, each clone was checked by Western blot. **Figure 4** shows the level of expression in several Kai-1 transfectants. Even though distinct bands are evident, most of the clones have protein in a wider size range (40-75 KDa) compared to the microcell hybrids. This is suggestive of more extensive Kai-1 protein post-translational modification, presumably heavy glycosylation. Kai/435.Pick-l and Pick-3 (lanes 1 and 3) have the least amount of Kai-1 protein while the Kai/435.subl.Picks 1 and 3 (lanes 5 and 7) show the highest levels. Lanes 6 and 8 represent Kai-1 transfectants which also have comparatively low levels of protein, however they were not inoculated into mice. As was seen with the neo11/435.E1 microcell hybrid, there was a morphological difference (size and shape) in culture between the transfectants with heavily modified Kai- 1 and those transfectants with protein bands separated by smaller size differences on the Western blot (data not shown). These data show that the amount of Kai- 1 protein expression varies greatly among transfectants and suggests that the level of expression may influence in vitro phenotypic characteristics. Furthermore, because the transfectants appear more heavily modified compared to the microcell hybrids, the Kai 1 expression from an introduced vector is qualitatively distinct from what is seen after introduction of a whole, normal chromosome 11.

Metastasis Assay of Transfectant Clones. We next tested whether Kai-1 transfectants showed reduced

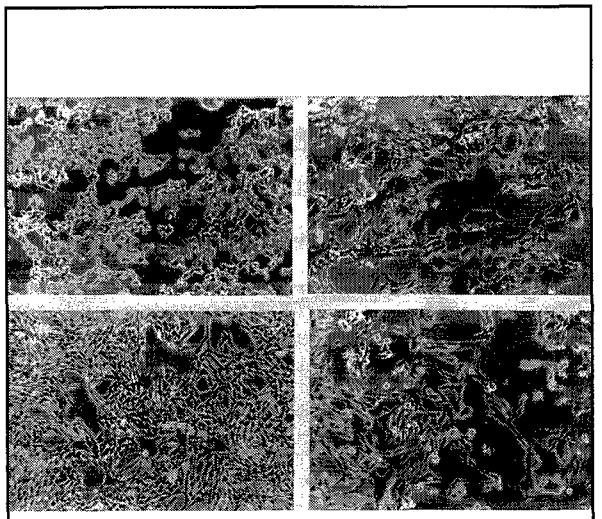


Figure 3. Photomicrographs (20X) of cells growing in culture: neo11/435.D1 (upper left), neo11/435.E1 (upper right), Kai-1 pick 3 (lower left) and Kai-1 pick 4 (lower right).

metastatic potential. Transfectant clones were inoculated into mammary fat pads of athymic nude mice and assayed for metastatic ability. **Table 1** lists the metastasis data for the parental cells, Kai-1 transfectants, chromosome 11 microcell hybrids, and neo transfectant controls.

As with most solid tumors, inherent heterogeneity for metastatic potential exists within the MDA-MB-435 parent cell population. We therefore isolated several single cell clones and assayed them for metastatic potential. Seven different subclones were each inoculated into at least eight mice. Even though the subclones had a wide range of numbers of lung metastases per mouse (0-167), the incidence of metastasis (86.6%) was about the same as that for the uncloned parental population (87.5%). The incidence of metastasis for the Kai-1 transfectant clones was not significantly different from the parental cells (83.8%), but number of lung metastases per mouse for Kai-1 transfectants (0-88) was lower than that of the parental cells (0-143 and 0-167). Incidence of metastasis (77.8%) and number of metastases in neo transfectant clones (0-37) was lower than in Kai-1 transfectants, but these were not statistically significant. The reduction is thought to reflect the early killing of mice due to large tumor burden. The only cells that had significantly fewer mice with lung metastases was the chromosome 11 microcell hybrid group (42.5%). This indicated that if the Kai-1 gene is important in controlling metastasis in the neo11/MDA-MB-435 model, it probably does not function alone.

Kai-1 Expression in Primary Tumors. Loss of the cDNA or failure to continue expressing Kai- 1 (McMaster et al., 1995) could explain the seeming disparity between the Western data and the lack of metastasis suppression in the Kai-1 transfectants. To test this, locally growing tumors were analyzed for Kai- 1 protein expression and compared to inocula. **Figure 5** shows a Western blot of inoculated cells and locally growing tumors for three Kai-1 transfectants and the MDA-MB-435 parental cells. The blot shows a decrease in the amount of Kai-1 protein in each transfectant primary tumor. Very little protein can be detected in the primary tumor or the metastatic lesions of the MDA-MB-435 cells, however the inoculated cells showed the same low level. We also analyzed the Kai/435.Pick 4 for Kai-1 levels in the lung metastases where we observed a further decrease. Preliminary data from a comparable analysis of three inoculated neo11/435 hybrids with matched locally growing tumor proteins showed equal or increased levels of Kai-1 for 2/4 hybrids and a slight decrease (< 2-fold) in the other hybrid (data not shown). These data suggest that Kai- 1 expression is reduced in vivo during tumor progression to metastasis.

Discussion

Our previous study showed that chromosome 11 harbors a strong metastasis-suppressor gene for the MDA-MB-435 cell line. Candidate breast cancer metastasis-suppressor genes on chromosome 11 are Kai-1, a prostate cancer suppressor gene, and TAPA-1, a gene which codes for a structurally related protein, respectively mapped to 11p11.2 and 11p15.5. Both express proteins which are TM4SF family members and could likely function in maintenance of cell integrity and adhesion (Nojima et al., 1993). Also, markers surrounding the mpa-1 locus show LOH in breast tumors (Ali et al., 1987), thus implicating the region for the presence of a suppressor gene. Furthermore, both prostate and breast tissues are hormonally regulated; hence, loss of hormonal regulation could result from the loss of activity of a common

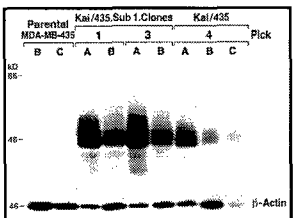


Figure 4. Western blot of matched sets of parental and three transfectant clones showing Kai-1 levels for inoculated cells (A), primary tumors (B) and lysates from metastatic lesions (C). Ten ug of protein were loaded per lane. Loading controls were determined on separate blots run identically and simultaneously with this blot, except that the sample was denatured before loading.

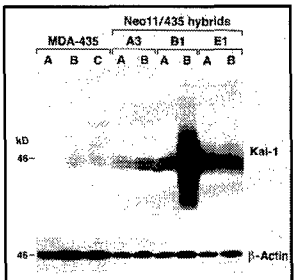


Figure 5. Western blot of matched sets of parental cells and three neo11/435 hybrids showing Kai-1 levels for inoculated cells (A), primary tumors (B) and metastatic lesions (C). Ten ug protein were loaded in each lane.

suppressor gene.

In this study, we wanted to determine whether either Kai-1 or TAPA-1 represented the operative metastasis-suppressor gene in our microcell hybrid cell lines. TAPA-1 appears to be a poor candidate as Western blot analysis revealed no obvious differences in the protein expression levels that correlated with metastatic potential. In contrast, Western blots showed that Kai- 1 levels inversely correlated with metastatic ability in the microcell hybrids. This result was consistent with the metastasis suppression resulting from transfer of normal human chromosome 11 and could be due to an increase in Kai- 1 protein expression.

To directly test the importance of Kai-1 as a metastasis-suppressor gene in breast cancer, we transfected full-length Kai-1 cDNA under control of a constitutive CMV promoter into MDA-MB-435. In theory, if Kai-1 were the key gene controlling metastasis, the same strong suppression of metastasis seen in the chromosome 11 MMCT hybrids should have been seen. In this study we could not clearly establish an effect of Kai-1 expression on metastasis. Kai-1 transfectants were not significantly suppressed for metastasis, but this may have been due, in part, to reduced expression in vivo. We could not eliminate the possibility that Kai- 1 levels dropped below a threshold level required for metastasis suppression. Kai-1 gene expression under the control of a stronger or inducible promoter may allow us to answer this question.

Another possible explanation for the failure of Kai-1 to suppress metastasis is altered post-translational modification. Most transfectants express proteins of higher molecular weights than those from microcell hybrids. Since chromosome 11 encodes other genes which might alter post-translational modification (e.g., fucosyl-transferase on 11q21)(McCurley et al., 1995), it is possible that multiple genes act in concert to exert such a strong effect on metastasis. However, additional studies will be required to establish correlations between Kai- 1 protein modification and metastatic potential.

The Western blot data suggest that levels of Kai-1 protein may be reduced as breast cells progress to metastatic ability. Non-tumorigenic 184 cells have higher levels of Kai-1 compared to the invasive and non-metastatic MDA-MB-436 cells, and these have higher levels than the metastatic MDA-MB-435 cells. Whether Kai- 1 expression uniformly falls upon transformation of breast epithelium will require more extensive studies.

Why do metastatic MDA-MB-435 cells express a reduced level of Kai- 1 protein? There may be loss of some trans-activating factor(s) necessary for expression, or expression could be altered by DNA modifications with the Kai-1 gene itself. In fact we have preliminary evidence that endogenous Kai-1 genes in metastatic MDA-MB-435 could be silenced due to methylation (Phillips and Weissman, unpublished observations). Demethylation experiments with 5-azacytidine could help clarify these data.

In summary, our results indicate that reduced expression of the Kai-1 metastasis-suppressor gene may contribute to the progression of breast cancer in humans. The modest decreases in metastasis observed in transfectants suggest that Kai- 1 is not acting alone to control breast cancer malignancy. It is not clear whether the weak suppression is due to a failure to maintain a threshold level of Kai-1 protein expression in vivo; whether the gene is altered; or whether protein is modified. More thorough analyses of the mechanisms mediating KAI-1 function will help to elucidate these possibilities.

Recommendations for follow-up experiments based upon these results

1. The lack of metastasis suppression in Kai-1 transfectants can be explained by loss of expression and/or altered glycosylation. While these conclusions is consistent with the data presented, we are dubious that Kai-1 is responsible for metastasis suppression. This is based partially upon intuition and reports of unpublished data that Kai-1 is not suppressive in human prostatic carcinoma. Therefore, we have opted to forego further studies of Kai-1. Since Kai-1 was found in prostate cancer and does not suppress in that model, we feel that it is a better expenditure of time to look elsewhere.

Section 5: ***Kai-1, a putative marker for metastatic potential in human breast cancer. Cancer Letters* (1997) in press**]

Summary

In collaboration with Dr. Lisa Wei, we attempted another strategy to assess the role for Kai-1 in breast cancer metastasis. Briefly, we measured Kai-1 expression level using Northern blotting. The text of the manuscript is printed below.

Abstract

The *Kai-1* gene maps to chromosome 11p11.2, is a metastasis-suppressor gene for human prostate cancer and also is involved in the progression of human pancreatic and non-small cell lung cancer . Recently, we showed that introduction of a single copy of normal, neomycin-tagged human chromosome 11 into highly metastatic MDA-MB-435 breast cancer cells suppressed breast cancer metastasis. Concomitantly, *Kai-1* levels were higher in chromosome 11/MDA-MB-435 cell clones. The purpose of this study was to test whether *Kai-1* expression is indicative of breast cancer metastasis using a panel of immortalized breast epithelial and breast cancer cell lines that represent multiple stages of breast cancer progression. Metastatic cell clones isolated from the parental mixed, wild-type population of MDA-MB-435 cells expressed the lowest levels of *Kai-1* mRNA; and chromosome 11 containing MDA-MB-435 (neo11/MDA-MB-435.A3 and neo11/MDA-MB-435. B1) cells had approximately twice as much *Kai-1* mRNA than the parental clones. MCF-10A, an immortalized normal-like nontumorigenic mammary epithelial cell line, had the highest level of *Kai-1* mRNA. We compared the metastatic propensity and invasive ability of a continuum of breast cancer cells with varying degrees of progression toward malignancy and found that these parameters tended to correlate inversely with *Kai-1* mRNA expression. These data suggest that, in addition to its role in human prostate, pancreatic, and non-small cell lung cancer, *Kai-1* may also be a useful marker for staging human breast disease.

Introduction

Breast cancer is one of the leading causes of death for women in the United States (Pisani et al., 1993). It is estimated that at least 184,000 new cases of breast cancer will be diagnosed and 44,000 women will die of the disease this year. If breast cancer is diagnosed and treated before spreading, 5 year survival approaches 100%. However, if metastases have developed, survival is poor. Therefore, metastasis is the most critical parameter determining patient survival from breast cancer.

Breast cancer progression is a multistep process resulting from a series of genetic changes (Sato et al., 1990). Metastasis is part of this progression and a late molecular event from initial tumor formation (Liotta, 1992). Breast cancer cells acquire metastatic capacity after several additional genetic defects. Characterization of the later molecular events in aggressive tumor cells can have several potential benefits including the development of new prognostic and therapeutic strategies in the treatment of breast cancer metastases. Although several genes associated with metastasis have been identified (Dear and Kefford,

1990; Liotta et al., 1991), the mechanisms of action of these genes and the ability to utilize these genes to discriminate metastatic from nonmetastatic cells still remains in its infancy.

Recently, we demonstrated that human chromosome 11 encodes at least one gene that significantly suppresses (> 95%) breast cancer metastases *in vivo* but does not inhibit tumor growth (Phillips et al., 1996). Therefore this activity can be easily distinguished from the tumor-suppressor genes encoded on chromosome 11 (Van Heyningen and Little, 1995). Our data in breast cancer cells followed those of Rinker-Schaeffer *et al.* who showed that microcell mediated chromosome transfer-introduction of chromosome 11 into Dunning rat prostate carcinoma cells suppressed metastasis (Rinker-Schaeffer et al., 1994). Subsequently, a prostate metastasis-suppressor gene, *Kai-1*, was cloned and mapped to human chromosome 11p11.2-13 (Dong et al., 1995). Following its initial description, *Kai-1* has been found to be involved in the progression of human non-small cell lung cancer, human pancreatic cancer and human prostate cancer (Adachi et al., 1996; Guo et al., 1996; Dong et al., 1996).

The purpose of this study was to determine whether *Kai-1* gene expression may also serve as a marker for metastasis in human breast cancer. Ten human breast cancer cell lines representing various stages of tumor progression were screened for the expression of *Kai-1* mRNA and compared to their estrogen receptor and progesterone receptor (ER/PR) status as well as the metastatic potential of each cell line. Our results indicated that loss of *Kai-1* gene expression may play an important role in the appearance of human breast cancer metastases.

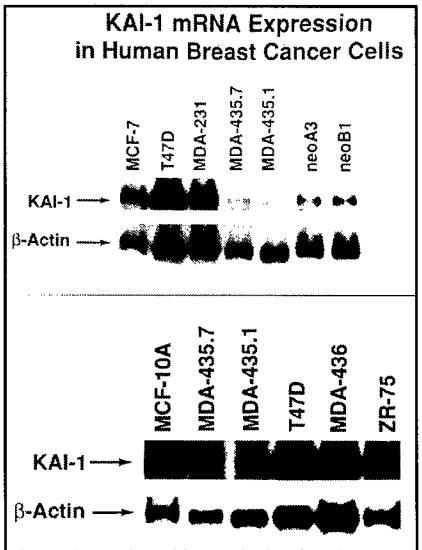


Figure 6. Northern blot analysis of Kai-1 gene expression in human breast cell lines. Total RNA(15 μg) isolated from human breast cancer cells: MCF-7, T47D, MDA-MB-231(A); ZR-75, MDA-MB-436(B); MDA-MB-435.1, MDA-MB-435.7, neo11/MDA-MB-435.A3 and neo11/MDA-MB-435.B1 (A&B) and RNA from an immortal mammary epithelial cell line MCF-10A(B) were separated on a 1% agarose/formaldehyde gel and hybridized with ^{32}P-labeled Kai-1 cDNA probe. The Kai-1 cDNA probe recognized a message of ~ 2.4 kb as estimated by RNA standards and ribosomal RNAs. As a loading control, β-actin levels also were monitored and are shown at the bottom of each blot. neo11/MDA-MB-435.A3 (neoA3) and neo11/MDA-MB-435.B1 (neoB1) are MDA-MB-435 cells containing a normal human chromosome 11.

Materials and methods

Cell Lines and Culture Conditions: The immortal normal-like human breast MCF-10A cell line (provided by Dr. Robert Soule, Karmanos Cancer Center) was grown in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 media supplemented with 5% horse serum, 0.5 μg/ml hydrocortisone, 12 μg/ml insulin, 0.02 ng/ml EGF and 1 ng/ml cholera toxin (Soule et al., 1990). Human breast cancer cell lines examined in this study included the following: MCF-7, ZR-75, T47D, MDA-MB-436, MDA-MB-231, MDA-MB-435, two-subclones of MDA-MB-435 from the wild-type population: MDA-MB-435.1 and MDA-MB-435.7, as well as two MDA-MB-435 clones with introduced normal human chromosome 11, neo11/MDA-MB-435.A3 and neo11/MDA-MB-435.B1. MCF-7, MDA-MB-436 and MDA-MB-231 cells were obtained from The Lombardi Cancer Center Tissue Culture Core Facility. ZR-75 and T47D cells were provided kindly by Dr. Dean Edwards (University of Colorado) (Elashry-Stowers et al., 1988). The parental MDA-MB-435 cells were a gift from Dr. Janet Price (U. T.-M.D. Anderson Cancer Center) (Price et al., 1990). Development and characterization of neo11/MDA-MB-435.A3 and neo11/MDA-MB-435.B1 were previously described (Phillips et al., 1996). MCF-7 cells were cultured in modified Improved Minimum Essential Medium (IMEM) without phenol red containing 5% fetal bovine serum (FBS). T47D and ZR-75 cells were maintained in Minimum Essential Medium (MEM) with 10% FBS and 6 ng/ml insulin. MDA-MB-231 cells were maintained in modified IMEM containing 10% FBS. MDA-MB-436, MDA-MB-435 and their subclones were grown in DMEM/Ham's F-12 media supplemented with 10% FBS. The neo11/MDA-MB-435 microcell hybrids were maintained in the same media with the addition of 600 μg/ml G418. All cells were incubated at 37°C, 5%

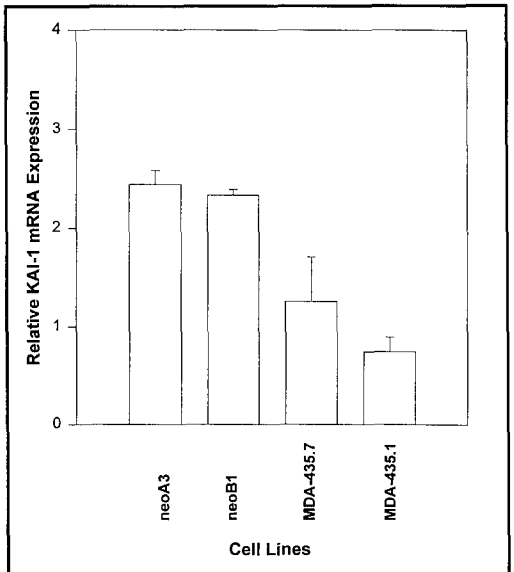


Figure 7. Relative Kai-1 mRNA expression in MDA-MB-435 derived human breast cancer cells. MDA-MB-435.1 and 435.7 are two subclones derived from the wild-type metastatic population of MDA-MB-435. neo11/MDA-MB-435.A3 (neoA3) and neo11/MDA-MB-435.B1 (neoB1) are MDA-MB-435 cells containing a normal human chromosome 11. Northern blot analyses were conducted in duplicate for each individual experiment. Hybridization signals for Kai-1 and β-actin levels were evaluated by densitometry and Kai-1 mRNA expression was normalized to β-actin levels. Data represent the mean values from duplicates. Values are representative of at least three independent experiments.

CO_2, in a humidified chamber. All cell lines were routinely tested and found to be negative for *Mycoplasma* contamination. The biological properties of these cell lines have been summarized elsewhere (Thompson et al., 1992).

Northern Blot Analysis: Standard techniques were used with minor modifications according to Wei *et al.* (Wei et al., 1988). Experiments were repeated at least three times using RNA isolated from different pools of exponentially growing cells. Cells were lysed in guanidine thiocyanate and total RNA were isolated by cesium chloride ultracentrifugation (Wei et al., 1988). Denatured total RNA (15 μg) were loaded onto each lane and fractionated by 1% agarose/formaldehyde gel electrophoresis then transferred to Gene Screen (Dupont-NEN Research Products, MA) via capillary blotting. Blots were pre-hybridized overnight and then hybridized in 50% formamide, 5x SSC, 5x Denhardt's solution and 1% SDS containing cDNA probe for 48-72 hrs at 42°C. A 1,030 bp *Kai-1* cDNA fragment generated by PCR was labeled by nick-translation with α-^{32}P-dCTP (Amersham). Blots were washed four times in 2x SSC, 0.5% SDS at room temperature, followed by washes in 0.2x SSC, 0.5% SDS at 52°C and then exposed to X-ray film with intensifying screens. As a control for equal loading and blotting of RNA, blots were stripped and rehybridized with a β-actin probe. The hybridization signals were quantified by densitometric scanning (The Discovery Series Software, PDI densitometer, NY).

Results

In order to evaluate the importance of *Kai-1* expression in the process of human breast cancer metastasis, we compared the *Kai-1* RNA levels in a panel of established breast cancer cell lines that represent varying stages of breast cancer progression. Estrogen and progesterone receptor status of each tested breast cancer cell line and the relative invasiveness and metastatic potential of each cell line are known. Invasiveness was assessed *in vitro* by the Boyden chamber chemoinvasion assay as reported by Thompson *et al.* and the relative metastatic potential evaluated by spontaneous metastasis assays in nude mice (Thompson et al., 1992).

Kai-1 mRNA expression was examined in cells maintained in their normal growth medium in the exponential growth phase. Northern blot analyses were performed using a nick-translated 1,030 bp probe containing the entire coding region for the *Kai-1* protein (Dong et al., 1995). A single *Kai-1* message transcript of ~ 2.4kb was detectable in all tested cell lines (**Figure 6**). Two clones derived from the wild-type metastatic population of MDA-MB-435, MDA-435.7 and MDA-435.1 (Phillips et al., 1996), had extremely low but detectable levels of *Kai-1* message. This was also found for the parental mixed population of MDA-MB-435 cells (data not shown). On the other hand, the relative *Kai-1* mRNA expression of two MDA-MB-435 cell clones containing an additional copy of human chromosome 11, neo11/MDA-MB-435.A3 and neo11/MDA-MB-435.B1, had a 2.4- and 2.3- fold higher level of *Kai-1* mRNA as compared to the parental control cells (**Figure 7**). MDA-MB-231 cells, reported to be highly invasive but modestly metastatic in athymic nude mice (Price et al., 1990), also had higher levels of *Kai-1* RNA than all the tested MDA-MB-435 clones (**Figure 6 top panel**). The MDA-MB-436 cells, invasive in *in vitro* assays and appear to be only locally invasive *in vivo* (Thompson et al., 1992), had approximately 8-fold higher *Kai-1* mRNA levels than the MDA-MB-435 cells (**Figure 6 bottom panel**).

Taken together, these studies indicated that *Kai-1* RNA expression correlated more tightly with metastatic potential than invasiveness. In addition, steroid receptor-containing breast cancer cells (ZR-75, T47D, MCF-7) generally had higher *Kai-1* RNA levels than receptor-negative cells (MDA-436, MDA-231, neoA3, neoB1, MDA-435.7, MDA-435.1) (**Figure 8**). There was one exception, MDA-MB-436 breast cancer cells (**Figure 8**). We also examined the level of *Kai-1* mRNA expression in the immortal normal-like breast epithelial cell line, MCF-10A, and found that these cells had the greatest *Kai-1* mRNA expression of all the tested cell lines (**Figures 6 bottom panel and 8**). MCF-10A cells are not tumorigenic (Soule et al., 1990). Like non-lactating normal breast tissue, MCF-10A cells do not express significant levels of steroid receptors. This is in contrast to breast tumors and breast cancer cell lines which express relatively high levels of steroid receptors (Pilat et al., 1996); therefore, the correlation between *Kai-1* mRNA levels and ER/PR status may not be the same in normal breast epithelium as in breast cancer specimens. Thus, these studies support a role for loss of *Kai-1* expression and may explain, in part, the progression from nonmetastatic transformed epithelial cells to metastatic lesions in human breast cancer.

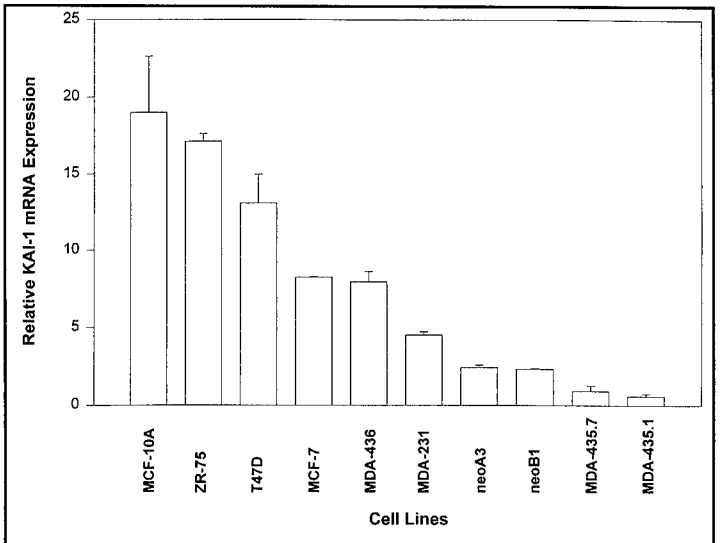


Figure 8. Relative Kai-1 mRNA expression in all human breast cells examined. Northern blot analyses were conducted in duplicate for each individual experiment. Hybridization signals were evaluated by densitometry and Kai-1 mRNA expression was normalized to β-actin levels. Data represent the mean values from duplicates and values are representative of at least three independent experiments.

Discussion

Metastasis, the most life-threatening aspect of cancer, is a complex multistep process that requires cancerous cells to escape the primary tumor, intravasate and extravasate the circulatory system, and then penetrate to peripheral sites and grow. Metastatic cells must have the capacity to adhere to basement membranes, degrade membranes, and be motile. Recently, we found that introduction of a normal human chromosome 11 into highly metastatic breast cancer cells suppressed metastatic ability (Phillips et al., 1996). From these studies, we concluded that a breast cancer metastasis-suppressor gene or genes exists on chromosome 11.

Kai-1, a prostate metastasis-suppressor gene, was identified and mapped to chromosome 11p11.2-13. The *Kai-1* gene is identical to CD82 and C33 genes (Dong et al., 1995). Interestingly, decreased *Kai-1* expression also correlates with poor prognosis in human non-small cell lung cancer, pancreatic cancer and prostate cancer (Adachi et al., 1996; Guo et al., 1996; Dong et al., 1996). These findings suggested that *Kai-1* expression might also serve as a marker in human breast cancer. To test this hypothesis, we measured mRNA expression in a panel of human breast cell lines that model stages of progression toward malignancy. We report that high RNA expression of *Kai-1* is a feature of many breast cancer cells with low metastatic potential and *Kai-1* RNA expression is substantially lower in the most aggressive and metastatic breast cancer cell lines.

In addition, two MDA-MB-435 cell clones into which an additional copy of human chromosome 11 had been introduced, were less metastatic and expressed consistantly higher levels of *Kai-1* m*RNA* expression than the parental MDA-MB-435 cells. While our data support the possibility that *Kai-1* expression may be important in human breast cancer metastasis, mRNA levels of *Kai-1* in both chromosome 11-containing hybrid clones were lower than that found in other tested breast cells. This

suggests that *Kai-1* expression may not be the single determining factor in controlling metastasis but that other factors are involved as well.

A role for *Kai-1* in controlling metastasis is initially very appealing. *Kai-1* belongs to a superfamily of transmembrane adhesion proteins known as TM4SF (Wright and Tomlinson, 1994). One family member, CD9 (approximately 54-65% identity with *Kai-1*) has been correlated with the metastatic potential of some breast cancer tumors and in non-small cell lung cancer (Miayake et al., 1995; Higashiyama et al., 1995). CD9's role in metastasis appears to be as a regulator of motility since overexpression of CD9 interferes in cell motility (Ikeyama et al., 1993). *Kai-1*'s role in metastasis, if any, may be further complicated by species and tissue specificity (Rinker-Schaeffer et al., 1994; Dong et al., 1995). For instance, Rinker-Schaeffer *et al.* reported that introduction of chromosome 11 into highly metastatic rat mammary tumor cells had no effect on metastasis (Rinker-Schaeffer et al., 1994) despite expressing more *Kai-1* (Dong et al., 1995). This differs from our results in which introduction of a human chromosome 11 dramatically suppressed human breast cancer metastasis. Transfection of *Kai-1* suppressed metastasis of rat prostatic carcinoma cells (Dong et al., 1995), but transfection into breast cancer cells gave equivocal suppression (unpublished observations). Thus, it is not yet known whether *Kai-1* directly suppresses human breast cancer metastasis. The precise mechanism of action of TM4SF family members, including *Kai-1*, remains unknown.

To our knowledge, these are the first findings demonstrating a possible role for *Kai-1* as a marker for human breast cancer metastasis. Based upon our results and upon its established role in prostate cancer, *Kai-1* gene expression in breast tumor tissues may be an important prognostic factor in predicting the likelihood of metastasis formation. Future studies will be directed to firmly establish the practical utility of *Kai-1* expression as a predictor of human breast cancer metastases.

Recommendations for follow-up experiments based upon these results

1. While these studies suggest that Kai-1 may be a useful marker for breast cancer progression, results are based solely on cell lines. While this observation suggests that Kai-1 may be useful in a clinical situation, this is not an immediate objective of our research. Since we are suspicious of an actual role for Kai-1 in breast cancer, further marker studies are low priority.

Section 6: **MMCT of pieces of chromosome 11 into MDA-MB-435 [Unpublished]**

Rationale

This section is based upon Specific Aim #1 of the original grant proposal. Our ultimate objectives are to map the gene(s) on chromosome 11 responsible for metastasis suppression in human MDA-MB-435 breast cancer cells and to understand how they work. We plan to develop a long-range map the metastasis-suppressor gene to within a 5 Mb region containing transcribable DNA flanked by two markers. From there, more refined mapping can be done using YAC, BAC, PAC or P1 contigs. Several different strategies were considered to accomplish this. We will briefly review the options with their respective advantages and disadvantages.

The MMCT experiments proposed could follow two tactics. Both are based on the fact that genes, whole chromosomes or chromosome fragments can be selectively transferred to mammalian cells. Our primary approach will be to prepare chromosome 11 microcell donors that have deletions (Bader et al., 1991; Dowdy et al., 1990). Radiation deletion mutants will be fused by MMCT to MDA-MB-435 followed by assessment of metastasis in athymic nude mice. Random deletions need not be mapped beforehand. They can be mapped following fusion based upon predetermined polymorphisms spanning chromosome 11. If the metastasis-suppressor gene is retained, functional complementation of the defect will be repaired and the cells will be nonmetastatic. If the gene has been deleted, suppression will not occur. Metastatic hybrids will be evaluated for portions of the chromosome 11 retained. Position of the

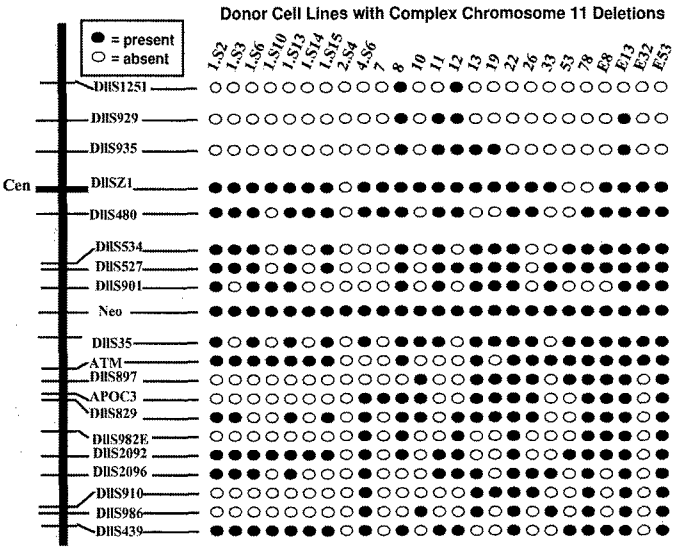


Figure 9: Map of chromosome 11 donors to be used in MMCT transfer experiments.

metastasis-suppressor gene can be inferred by the smallest region of shared deletion. This has most recently been used to positionally clone tumor suppressor genes on chromosome 17 for prostate cancer (Murakami et al., 1995) and breast cancer (Plummer et al., 1997).

The second approach is to utilize MMCT donors with previously defined fragments of chromosome 11 (Chen et al., 1995). The advantage of this approach is that fully-defined DNA is introduced into the cells. While aesthetically pleasing, the time required to fully characterize the donor chromosome fragment can take months to years. This second approach was to be a contingency; however, we have initiated a collaboration with Drs. Jane Fountain (University of Southern California) and Gavin Robertson (Ludwig Cancer Center, University of California-San Diego) to map melanoma tumor suppressor genes from chromosome 11. Dr. Fountain, in collaboration with Drs. Tracy Lugo (now at NIH) and Robertson made a panel of chromosome 11 donors which we now have in our laboratory. Therefore, we have begun making hybrids using these reasonably well-characterized donors. We are prepared to make additional donors; however, this strategy appears to be better suited to our current capabilities. A schematic diagram of the chromosome 11 donors is shown in **Figure 9**.

Methods

Preparation of microcell-tumor cell hybrids: Mouse A9 cell lines containing human chromosome 11 (MCH556.1) will be used to prepare microcells according to standard methods (Weissman and Stanbridge, 1983; Saxon et al., 1986; Trent et al., 1990). Briefly, semiconfluent A9 cells (Murine fibrosarcoma cells widely used for preparing microcells because they are highly efficient at accepting new DNA and they readily form micronuclei) are treated with colcemid prior to addition of cytochalasin B to form micronuclei. For fusion to tumor cells, isolated microcells are fused with brief exposure of phytohemagglutinin-pretreated tumor cells to polyethylene glycol. After the PEG is removed, the medium is replaced for 16-24 hr in order to allow the cells to recover. G-418 is added to select for variants which contain the added chromosome with neo marker. Individual colonies can be isolated with cloning rings or pooled then cloned (after approx. 14-42 days). Pooling followed by random cloning minimizes unintentional selection. It is anticipated that clones (20) will be expanded in tissue culture in G418-containing medium, frozen, and studied further.

Preparation of chromosome 11 fragments. If needed, we will use standard methods to prepare the deletion mutants (Dowdy et al., 1990; Cox et al., 1990). Briefly, microcells with intact chromosome 11 containing an integrated neo marker will be prepared using MCH556.1 Purified microcells will be irradiated with 2-3 Gy using a γ-irradiation source.

Screening and hybrid characterization. Prior to preparing radiation deletion microcells, we will first identify 50 polymorphic microsatellite markers spanning chromosome 11. Unequivocal detection of a different allele in hybrids will indicate that the added chromosome is present and that the marker has not been deleted in the microcell donor. Absence of an allele that was present in MCH556.1 indicates that the

marker was deleted before transfer. These 50 polymorphic loci will be chosen because their location on chromosome 11 is well-defined; because they are relatively evenly dispersed over the chromosome [representing an average distance between markers of 2.0 Mb (100 Mb ÷ 50 markers) (Litt et al., 1995). This is less than our goal of 5 Mb.].

Mapping the metastasis-suppressor gene Hybrids will be concurrently injected into separate mice with the following controls: parental MDA-MB-435 cells (negative control) or neo11/MDA-MB-435.B1 cells (positive control). Metastases will form whenever the metastasis-suppressor locus is absent. Alignment of the chromosome fragments will reveal a commonly deleted region (failure to amplify the "donor" allele) in metastatic hybrids. This will map the coordinates of the metastasis-suppressor gene. It is possible that more than one metastasis-suppressor locus is encoded on chromosome 11. If this is the case, clusters of deletions will be found.

Results

Identification of polymorphic markers on chromosome 11 that are informative for MDA-MB-435 MMCT. One of the baseline pieces of data required for assessing whether or how much chromosome 11 has been retained in MMCT hybrids is dependent upon our ability to distinguish donor from recipient chromosome 11. To do this, we have employed a PCR-based assay to amplify polymorphic markers spanning chromosome 11 (Weber, 1990a; Weber, 1990b; Weber and May, 1989). We have identified 19 informative markers (D11S-865,-876,-922,-925,-929,-931,-956,-975,-1259,-1300,-1311,-1316,-1323,-1333,-1340,-1367,-1384,-PYGM, INT2) from 26 primer pairs evaluated. The panel is biased for 11q; however, 6 polymorphic markers for 11p were identified. Additional markers are being evaluated.

MMCT of chromosome 11 fragments into MDA-MB-435. Several additional hybrids have been generated, frozen and DNA is being prepared for pre-injection characterization. We have also successfully (pending confirmation) introduced chromosome 11 fragment clone 7 shown in **Figure 9**. This chromosome 11 fragment was chosen because of preliminary data we have collected with Dr. Jane Fountain. This fragment apparently encodes a melanoma tumor suppressor gene. Since this region coincides with one commonly involved in breast cancer progression, we decided to try MMCT of this fragment early in the series.

Summary of findings and recommendations for follow-up experiments

Progress on Specific Aim 1 has somewhat been slower than anticipated for two reasons. First, the radiation source for making the chromosome 11 deletion variants was unexpectedly out of service for several months awaiting safety evaluation and upgrading. Thus, we had to delay beginning experiments. Second, the person who was primarily responsible for beginning these studies was technically unable to do MMCT. Therefore, Dr. Hwang was replaced by Dr. Seraj and Ms. Deana Hicks. Both have learned the MMCT procedure and have made some hybrids. However, the hybrids have not yet been characterized. We hope that progress on this aim will proceed more efficiently in the upcoming year.

Section 7 **Use of microdissection to help map regions of chromosome 11 deleted in breast cancer [Unpublished]**

Rationale

The data suggesting that chromosome 11 encodes a metastasis-suppressor gene, while dramatic, are crude. Efforts to refine the region on chromosome 11 where we search for the gene(s) would significantly improve chances of success. Under the auspices of a separate program project grant from NCI, we obtained a small number (50 samples) of breast cancer tissues from the Karmanos Cancer Center representing different stages of disease progression (i.e., localized *vs.* metastatic). Most of these cases have more than 10 years follow-up clinical history, making interpretation even more straightforward. The

samples were provided in a blinded manner under the auspices of an NIH program project grant on which we were investigators.

Using the technique of microdissection developed by Emmert-Buck and colleagues (Zhuang et al., 1995b; Zhuang et al., 1995a), we are performing LOH studies in an effort to better define the region(s) on chromosome 11 present/absent in metastatic disease. The technique entails dissection of individual cells from a 10-12 um section, isolation of DNA and PCR of polymorphic markers spanning the chromosome.

Summary of findings and recommendations for follow-up experiments

We have no publishable data yet to report; however, we have been able to successfully isolate DNA from sections and PCR amplify. We are doing these studies in collaboration with Dr. Ron Grenko in the Division of Anatomic Pathology as time permits. Progress has been slow since this is not a top priority. Nonetheless, this rather small investment in time and energy may allow us to make a significant jump in identifying the crucial regions of interest.

Section 8 **Use of differential display to identify metastasis-suppressor genes on chromosome 11 [Unpublished]**

Rationale

This section corresponds to Specific Aim 3 of DAMD-17-96-1-6152. The purpose is to directly identify genes expressed more in nonmetastatic neo11/hybrids compared to metastatic cells. Two methods for accomplishing this — differential display and/or subtraction hybridization — have already been used by us to identify metastasis-suppressor genes in melanoma. Assuming quality mRNA can be obtained, success of both techniques depends upon relatedness and relative homogeneity of the populations. Whenever populations are chosen for which a minimal number of phenotypic differences exist and for which minimal diversification (i.e., genomic or phenotypic instability) has occurred, the likelihood that differences observed are those being sought increases significantly. We originally chose 435.1 as the metastatic population for these studies and neo11/MDA-MB-435.B1 (11.B1) as the nonmetastatic clone. Both are recently derived clones; therefore, they are still relatively homogeneous. Both lines are related — the only genetic difference is introduction of chromosome 11 — yet they represent the "extremes" in metastatic potential within the MDA-MB-435 system. The other limitation of these techniques is that they are suitable for detecting levels of expression; however, they may not pick up differences causing loss of function (e.g., mutation). Likewise, events downstream of mRNA expression may not be properly evaluated. Despite these potential limitations, the potential for a quantum leap in our understanding of gene(s) regulation breast cancer metastasis is quite high.

Subtractive hybridization requires purification of mRNA from metastatic and nonmetastatic cells. After enrichment, cDNA libraries are constructed (approximately 10^6-10^7 λZAP II plaques containing average insert size of 1.0-1.5 kb). These libraries are amplified once to make large stable quantity of high titre stock representing 90->99% of mRNAs produced by our cells. Single round amplification will minimize the chance that slowly growing clones will be underrepresented in large-scale amplification. Heteroduplexes prepared from first strand 11.B1 cDNA and 435.1 biotinylated mRNA are reacted with streptavidin before extraction using phenol:chloroform. Unbound single strand cDNA is the subtracted library and can also be used for probing blots and attainment of full-length cDNA if needed. Only cDNAs resulting from nonmetastatic cells exclusively or those found in greater abundance (our initial criteria will be >10-fold) in nonmetastatic cells compared to 435.1 cells will be evaluated further. Full-length cDNA clones (Full-length is defined by translation initiation sequences with flanking Kozak code and suitable open-reading frame determined by BLAST analysis. cDNA size should be similar to the transcript size detected by Northern blot.) are then re-cloned into expression vectors (for large-scale production of the putative metastasis-suppressor genes).

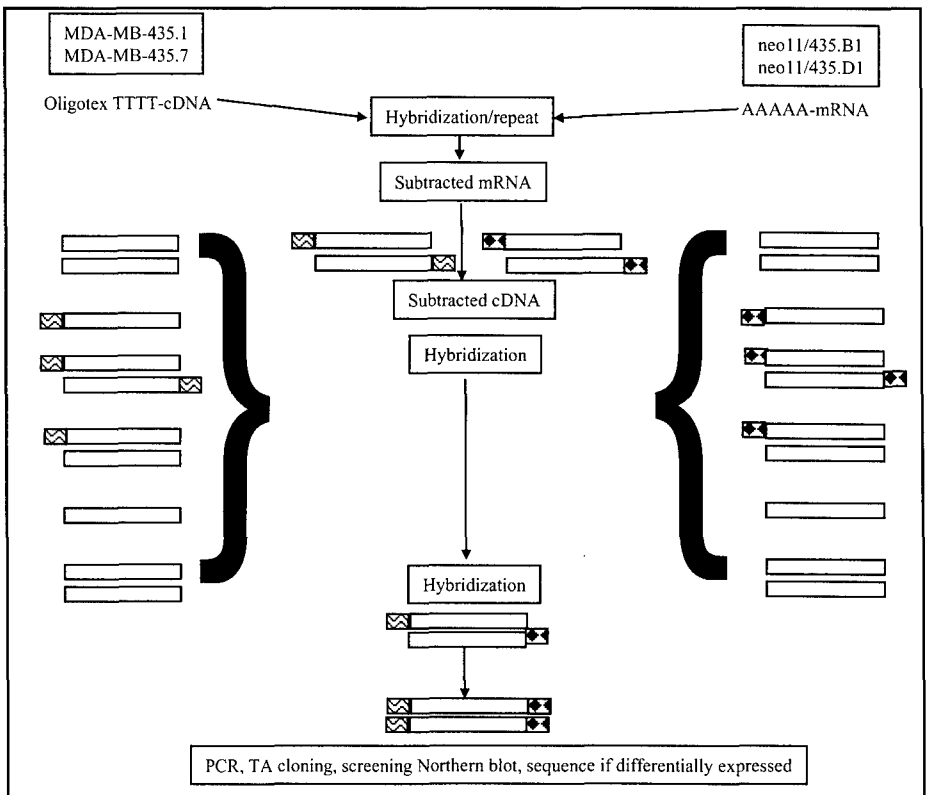


Figure 10. Schematic diagram of RDA and DD-RT-PCR techniques used in this research

Differential display (Liang and Pardee, 1992; Liang et al., 1993; Watson and Fleming, 1994) uses reverse transcription followed by PCR to produce uniquely-sized cDNA fragments from complex mRNA populations. By using radiolabeled dNTPs during the amplification procedure, 100-500 bp fragments can be produced from a primer set consisting of oligo-dT (3') and random primer sets (5'). The fragments can then be visualized using sequencing gels and fluorography. Theoretically, twelve oligo-dT primers (T_{12}MN where M may be dG, dA or dC and N can be dA, dC, dG or dT) in all combinations with at least twenty different random 10-mers (Operon) would be sufficient to display cDNAs from 10,000 different mRNA species. This technique has been previously used to identify maspin (Zou et al., 1994) and α6 integrin (Sager et al., 1993) as putative tumor suppressor genes and other sequence-tagged sites (Watson and Fleming, 1994) in human breast cancer.

DD-RT-PCR, while powerful, is susceptible to false positive results. This is most likely due to the sensitivity of the PCR reaction. Using a strategy developed by us in the melanoma cells, false leads are eliminated before large blocks of time and reagents have been expended. The RT-PCR reaction is repeated with the same primers which identified a differentially expressed band. Approximately half of the bands fail to amplify or show significant quantitative differences in an independent reaction. Each reaction is performed with separate mRNA preparations collected on different days. The rationale is that important differences will not be as dependent upon confluence or other "trivial" technical considerations. Bands that are reproducible are excised, end-labeled and used to probe RNA blots using a panel of relevant cells. This "screening Northern" tests only a portion of cell lines for desired patterns of expression (i.e., highest in nonmetastatic hybrids). Following a suitable result with the screening Northern, testing on a full panel of cells (i.e., all MDA-MB-435 hybrids) is done. By this time, our experience is that >75% of sequences are no longer of sufficient interest for further follow-up. Once a suitable probe has been found, full-length cDNA is obtained by screening previously prepared libraries from 11/.B1 cells. Length of the cDNA must be within 10% of the transcript size predicted by Northern blot.

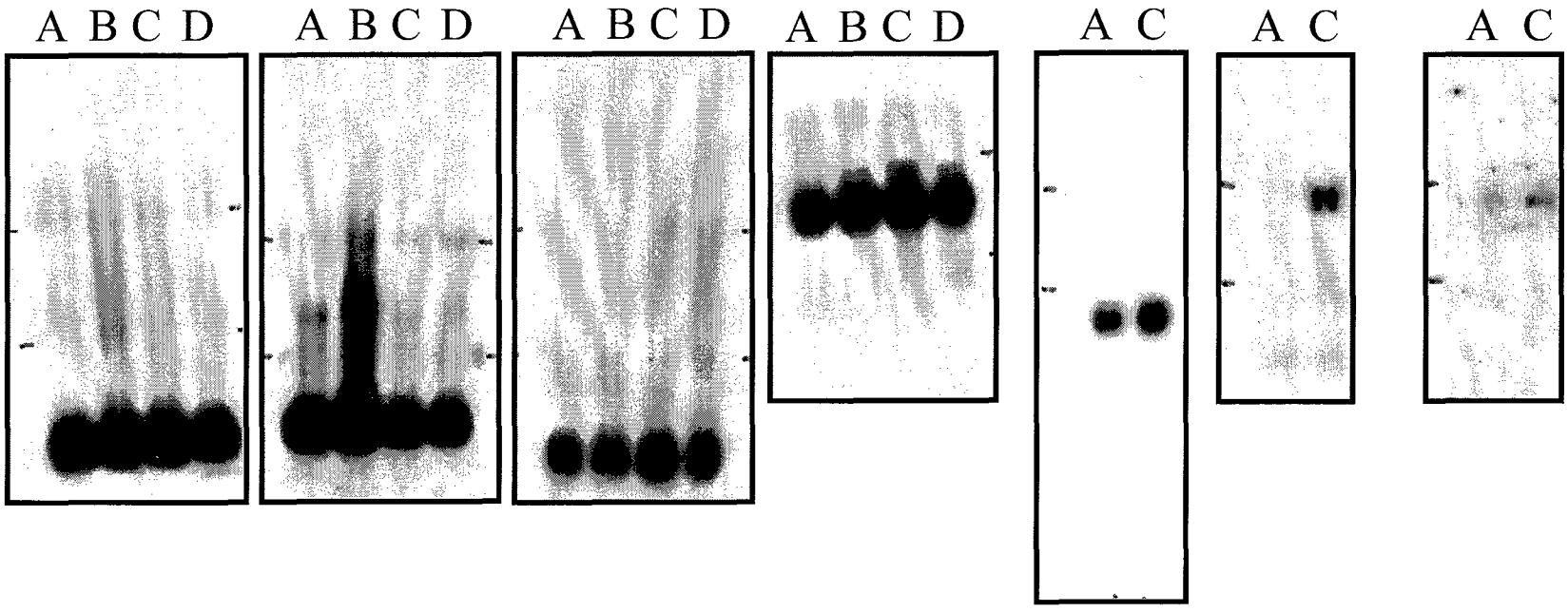
Priority of cDNA sequences for follow-up highest priority will be unique (<50% homology) cDNA sequences expressed exclusively in nonmetastatic hybrids. The majority of metastasis-suppressor genes display quantitative differences in expression, rather than qualitative. Therefore, only cDNAs that detect

quantitative differences >10-fold will be chosen for follow-up.

Results

Differential display (DD-RT-PCR), and the related representational differential analysis (RDA), were performed as described above with 435.1 and 11.B1. RDA was chosen as a modified strategy because it apparently produces fewer false positives than standard DD-RT-PCR (Lisitsyn, 1993). A schematic diagram of the experimental procedure is shown in **Figure 10**. Several candidate cDNAs (expressed exclusively or substantially more in MMCT hybrids) were identified. Follow-up Northern blotting experiments eliminated most candidates from further consideration. Therefore, a more extended panel was chosen for subsequent experiments.

This second panel included 435.1, 435.7, 11.B1 and 11.D1. The results from this extended panel provided sufficient evidence that many of the initial false-positives had been alleviated. Several representative Northern blots are shown in **Figures 11-18**. These figures show that some of the candidate cDNAs were not differentially expressed (cl.154, cl.157, cl.113, cl.65, cl.24,); whereas, others revealed quantitative differences between cell lines (cl.7, cl.33, cl.21, cl.77, cl.78, cl.10). Some of these results were corroborated in an extended panel of cells, but the pattern of expression was not verified for others (**Figures 13-15, 17, 18**).



E96-334	E96-334	E96-334	E96-333	E96-334	E96-334	E96-334
poly(A)+	poly(A)+	poly(A)+	poly(A)+	poly(A)+	poly(A)+	poly(A)+
amt. Loaded - 2.5 ug	amt. Loaded - 2.5 ug	amt. Loaded - 2.5 ug	amt. Loaded - 2.5 ug	amt. loaded 2.5	amt. Loaded 2.5	amt. Loaded 2.5ug
Probe: cl.154	Probe: cl.157	Probe: cl.113	Probe: cl.65	Probe: cl.7	Probe: cl.33	Probe: cl.21
1.0 kb	1.2 kb	1.0 kb	3.0 kb	1.5 kb	4.7 kb	4.5 kb
NDE, NSH	NDE, NSH	Expression 2X, NSH	NDE, NSH	Expression 2X NSH	Expression >10X Homology: MuLV?	Expression >5X Not sequenced

LEGEND

A MDA-MB-435.1
B MDA-MB-435.7
C neo11/435.B1
D neo11/435.A3

NDE - no difference in expression
NSH - no significant homology

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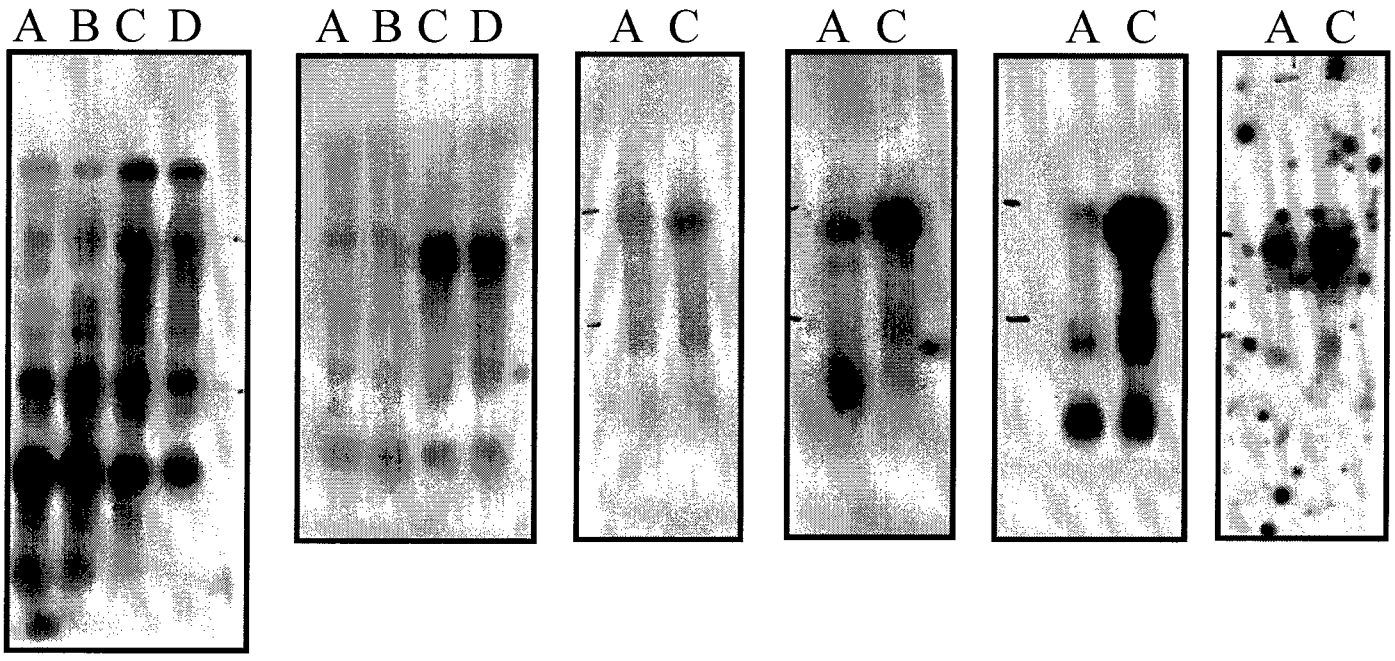
Figure 11



E96-207	E96-207	E96-018	E96-018	E96-310	E96-310
poly(A)+ amt. Loaded 2.5 ug Probe: C24 1.3 kb NDE, NSH	poly(A)+ amt. Loaded 2.5 ug Probe: cl.77 4.3, 4.7 kb Expression >10X Homologies: CSF receptor (67.7% over 189 bp) TNF-alpha (71.2% over 104 bp) integrin platelet fibrinogen receptor (53.8% over 160 bp)	poly(A)+ amt. Loaded 2.5 Probe: cl.77 4.3, 4.7 kb See 96-207	poly(A)+ amt. Loaded 2.5 Probe: cl.78 4.3 kb Expression >10X but not different in expanded Northern blotting panel NSH	poly(A)+ amt. Loaded 2.5 Probe: cl.33 4.3 kb Expression >10X Homology - MuLV	poly(A)+ amt. Loaded 2.5 ug Probe: cl.21 1.0 kb Expression >5X NSH

LEGEND

A MDA-MB-435.1
B MDA-MB-435.7
C neo11/435.B1
D neo11/435.A3

NDE - no differential expression
NSH - no significant homology

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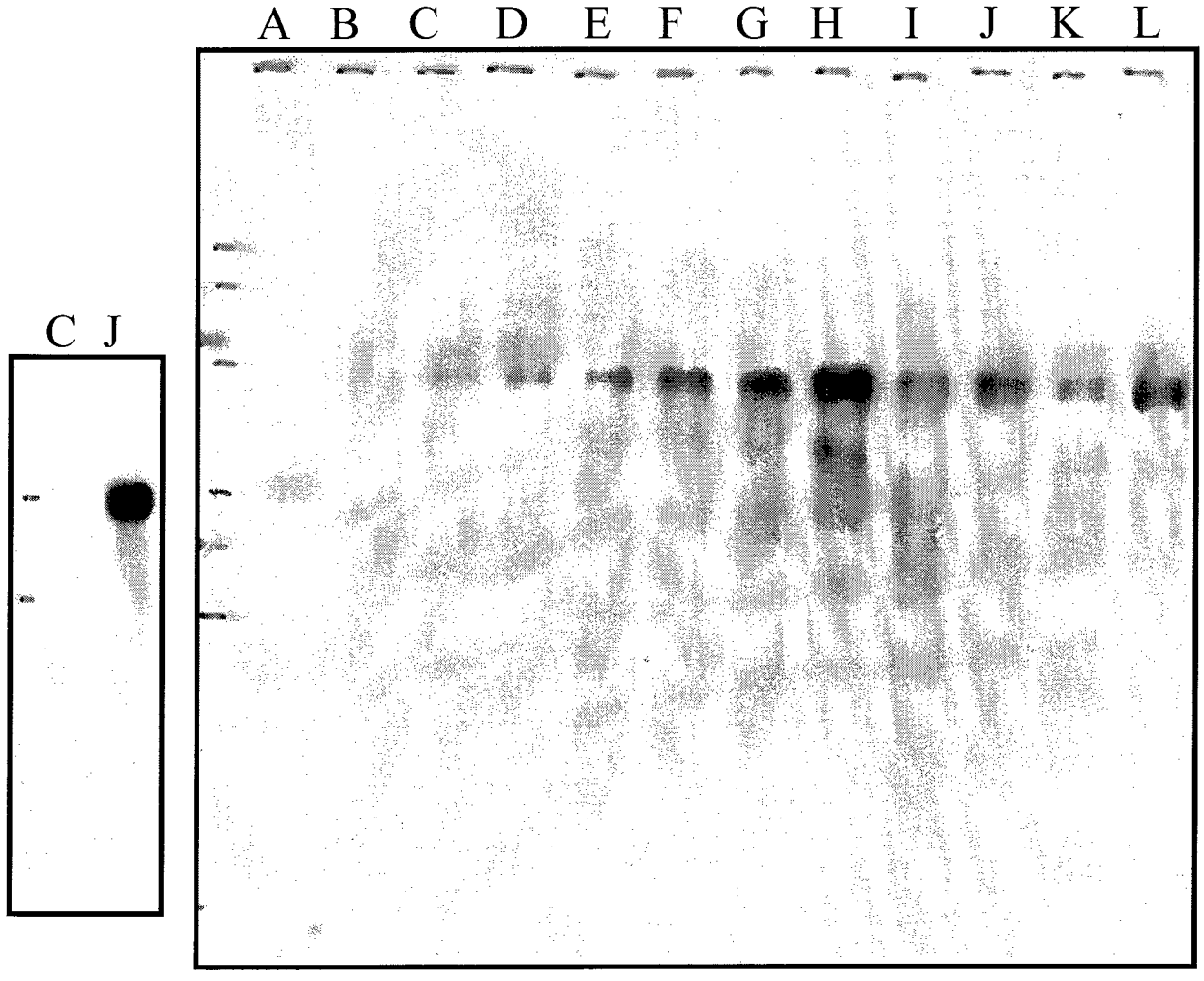
Figure 12



LEGEND

A	mRNA markers
B	MDA-MB-435
C	MDA-MB-435.1
D	MDA-MB-435.7
E	neo6/435.A1
F	neo6/435.C1
G	neo6/435.E2
H	neo6/435.G1
I	neo11/435.A3
J	neo11/435.B1
K	neo11/435.D1
L	neo16/435.E2

E95-337

poly(A)+
amt. Loaded 2.5 ug
Probe: cl..10
4.0 kb
Expression 3X
Homology - not tested

E96-334

poly(A)+
amt. Loaded 2.5 ug
Probe: cl.10
4.7 kb
Expression >10X
NSH

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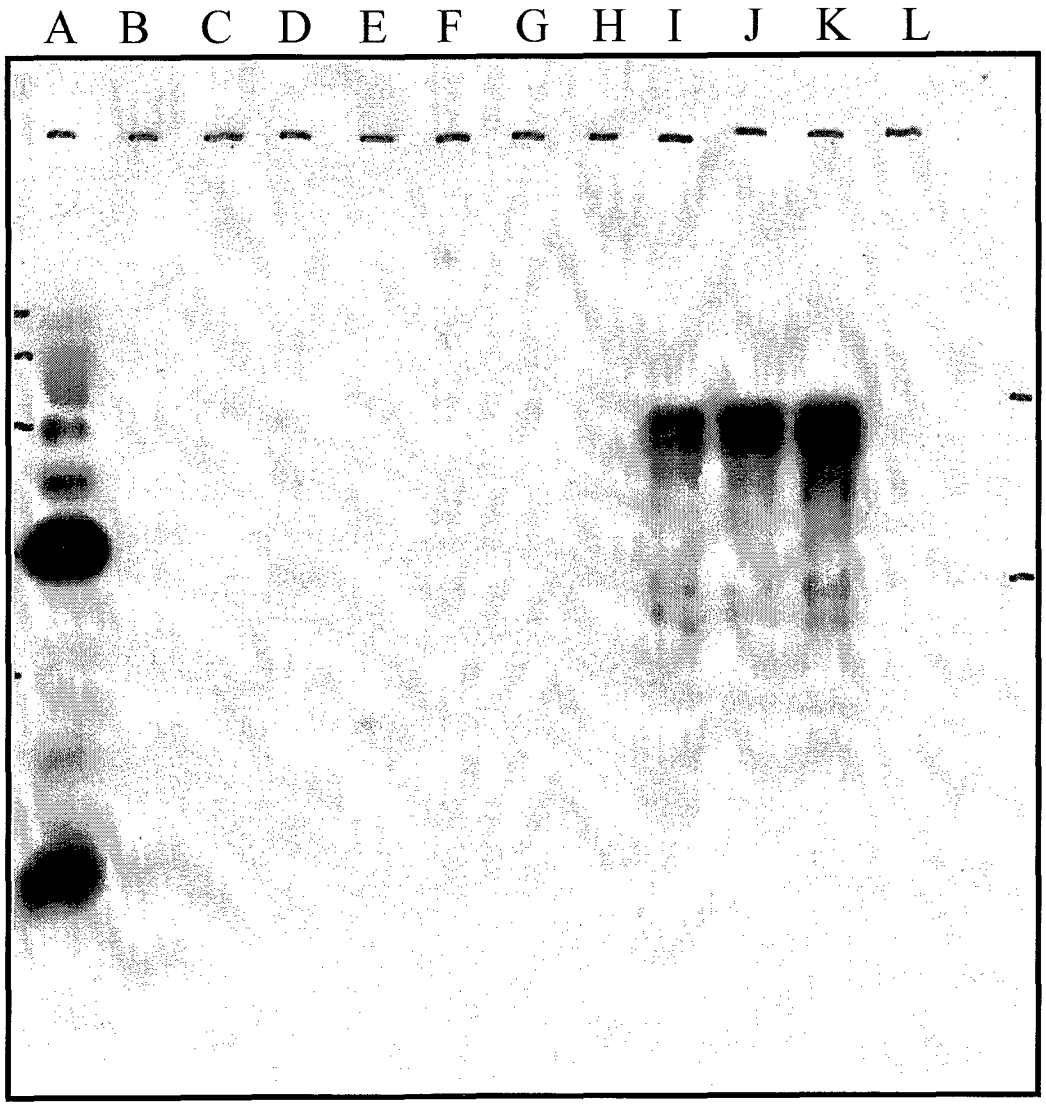
Figure 13



A mRNA markers
B MDA-MB-435
C MDA-MB-435.1
D MDA-MB-435.7
E neo6/435.A1
F neo6/435.C1
G neo6/435.E2
H neo6/435.G1
I neo11/435.A3
J neo11/435.B1
K neo11/435.D1
L neo16/435.E2

E95-007

poly(A)+
amt. Loaded 2.5 ug
Probe: cl..53
4.2, 4.5 (doublet) kb
Expression >10X
Homology - suspect MuLV
by size comparison to previous experiments

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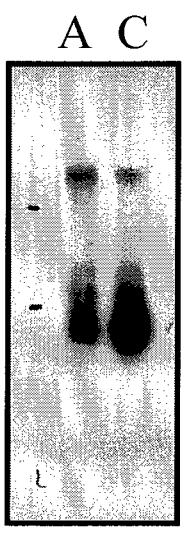
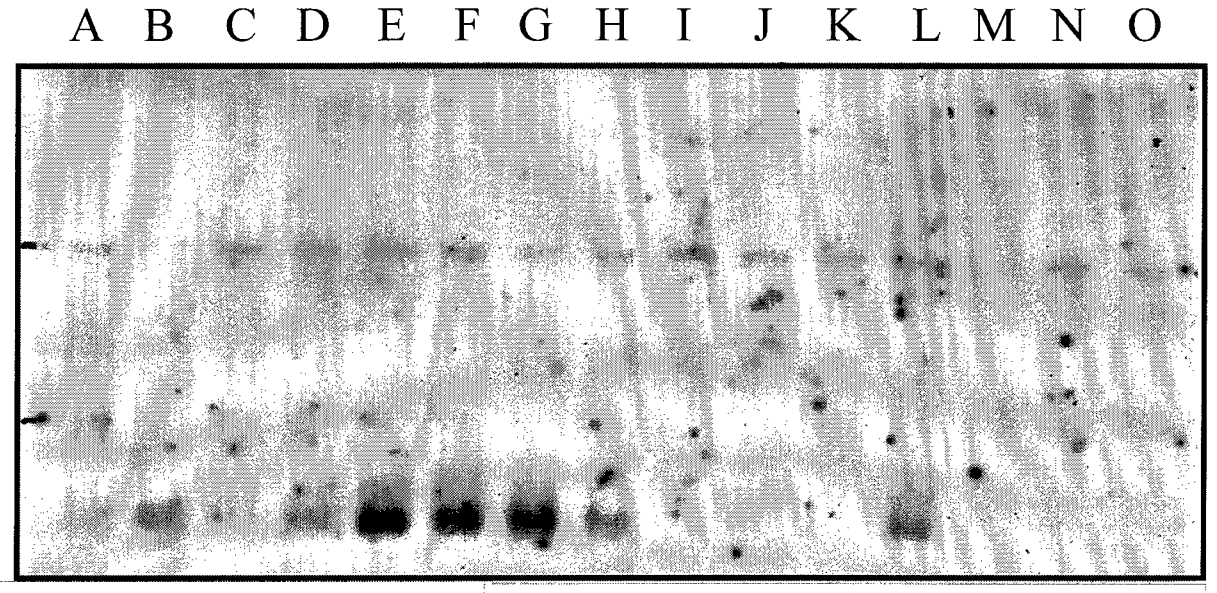
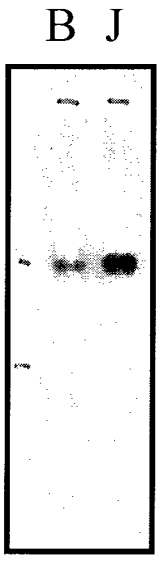
Figure 14







E96-310

poly(A)+
amt. Loaded 2.5 ug
Probe: cl..27
1.0 kb
Expression 5X
Homology not tested

E96-310

poly(A)+
amt. Loaded 2.5 ug
Probe: cl..27
1.0 kb
Expression 5X
Homology: human epsilon globin; maps to chromosome 11 but usually expressed only in embyrogenesis

LEGEND

A MDA-MB-435
B MDA-MB-435.1
C MDA-MB-435.7
D neo11/435.A3
E neo11/435.B1
F neo11/435.D1
G neo11/435.E1
H neo6/435.A1
I neo6/435.C1
J neo6/435.E2
K neo6/435.G1
L neo16/435.E2
M neo11/A9 (donor)
N neo11/C8161.4
O neo11/C8161.5b

E96-124

poly(A)+
amt. Loaded 2.5 ug
Probe: cl.27
4.7 kb
Expression 4X
Homology not tested

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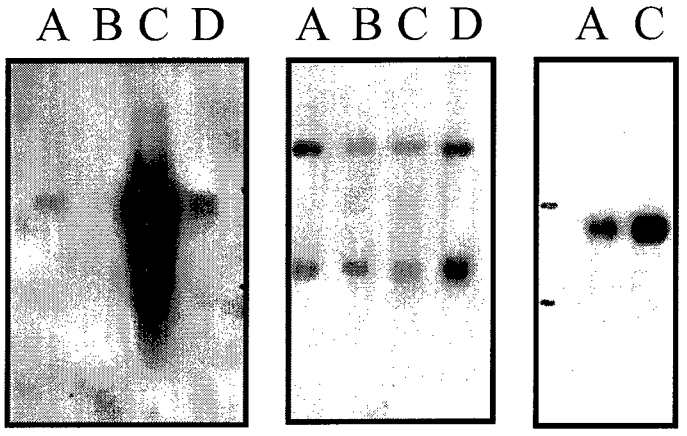
Figure 15



LEGEND
A MDA-MB-435.1
B MDA-MB-435.7
C neo11/435.B1
D neo11/435.A3

NDE - no difference in expression
NSH - no significant homology

E96-333

poly(A)+
amt. Loaded 2.5 ug
Probe: cl.20
4.7 kb
Expression inconsistent
in other experiments
NSH

E96-333

poly(A)+
amt. Loaded 2.5 ug
Probe: cl.17
2.0, 6.5 kb
NDE
Homology not tested

E96-124

poly(A)+
amt. Loaded 2.5 ug
Probe: cl.6
3.7 kb
Expression 3X
Homology - Human elongation factor 2
(91.9% over 135 bp)

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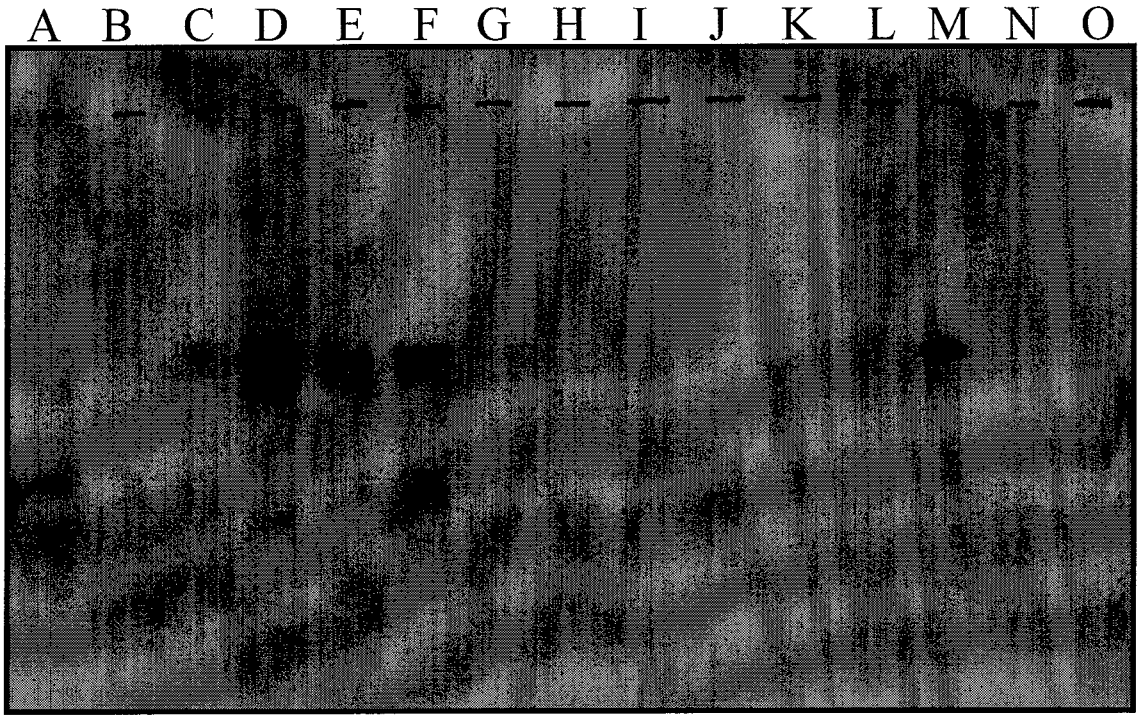
Figure 16



E96-284

poly(A)+
amt. Loaded 2.5 ug
Probe: cl..77
3' probe

LEGEND

A	MDA-MB-435
B	MDA-MB-435.1
C	MDA-MB-435.7
D	neo11/435.A3
E	neo11/435.B1
F	neo11/435.D1
G	neo11/435.E1
H	neo6/435.A1
I	neo6/435.C1
J	neo6/435.E2
K	neo6/435.G1
L	neo16/435.E2
M	neo11/A9 (donor)
N	neo11/C8161.4
O	neo11/C8161.5b

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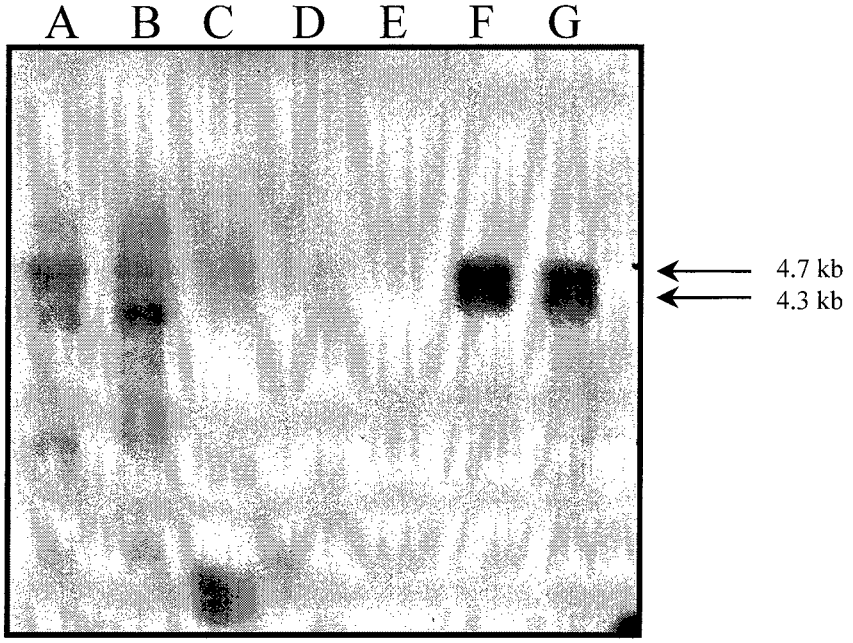
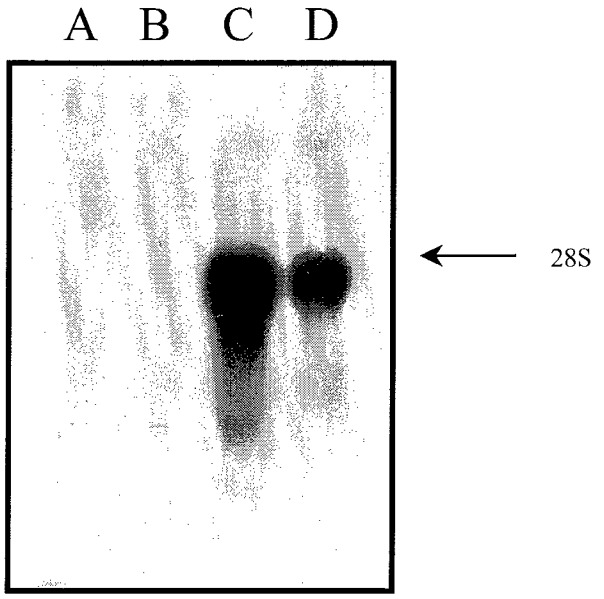
Figure 17



A MDA-MB-435.1
B MDA-MB-435
C MDA-MB-231
D MCF-7
E MCF-10A1
F neo11/435.E1
G neo11/435.B1

E96-327

poly(A)
amt. Loaded 5 ug
Probe: cl.77-111 (1.0 kb Ec/Sa)
Homology - none noted



A MDA-MB-435.1
B MDA-MB-435.7
C neo11/435.B1
D neo11/435.E1

E96-251

poly(A)+
amt. loaded
Probe: cl.77-111 (Ec/Sa)
1.0 kb

Welch, D.R.
DAMD17-96-1-6152
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Figure 18

The most interesting candidate was cl.77. Expression was exclusively in neo11/435 hybrids and the A9 cell donors (**Figures 17-18**). Two bands approximately 4.3 and 4.7 kb were detectable. The 300 bp cDNA was sequenced and no significant homology to known DNA was observed in a BLAST search. Cl. 77 cDNA was then used to screen a cDNA library generated from 11.B1. Several longer cDNAs were obtained and used to screen Northern blots. Similar patterns of expression were observed. Therefore, cl.77 variants appeared to be *bona fide* candidate metastasis-suppressor genes by expression pattern.

Two different sized clone 77 cDNAs were obtained (designated cl.77-111 and cl.77-211). The sizes of these cDNAs were 6.3 kb and 1.4 kb, respectively. Both cDNAs were sequenced and subjected to BLAST analysis. The 5' and 3' ends of the molecule were unique sequences; however, as one proceeded internally, homologies began to emerge. Both cDNAs exhibited regions of homology to murine leukemia virus retroviral sequences. There were still unique sequences present, however none appeared to be a *bona fide* open reading frame. While this is intriguing, we decided not to pursue this finding further since we suspect that it is an artefact of the MMCT technique. That is, the chromosome 11 donor used for our experiments carries neomycin resistance because of an integrated retrovirus based upon MuLV. We have not proven that this is exclusively the case, but all of the circumstantial evidence suggests this. Several other candidates were sequenced and contained MuLV sequences as well (dot blot analysis with MuLV probe). Having exclusive expression in cell lines containing the MMCT donor is consistent with this conclusion.

Preliminary experiments are currently being conducted to perform the PCR reactions so that the MuLV sequence amplification will be minimized. An example of technique modification is the incorporation of "spiked" MuLV DNA in MDA-MB-435 PCR reaction mixtures. As of mid-June 1997, we believe that we have overcome this hurdle; however, a few more experiments are required.

The differential expression of MuLV sequences may be providing a clue regarding suppression. One possibility is that the long terminal repeats (LTR) are facilitating transcription of adjacent genes. Unfortunately, LTRs are extremely potent, making it difficult to predict the distance from the integration site where they may be exerting a transcriptional effect. While this may be biologically interesting, we have decided not to pursue this avenue since it does not adequately fit into our long-term objectives.

Summary of findings and recommendations for follow-up experiments

1. Differential display and RDA represent the greatest "home run" approach; therefore, we will continue with this strategy. Dr. Hwang did a lot of work on this project. For reasons not elaborated, there were concerns with his ability to carry out this project further. Despite close oversight, he continued to make imprudent decisions. This particular project will be taken over by Dr. Seraj. Pilot studies performed by Dr. Seraj have been encouraging. He has obtained larger PCR products, making subsequent studies a little more straightforward. The major technical advance will be incorporation of screens to eliminate MuLV sequences from follow-up.

2. Our experience in the melanoma system causes us to question the use of the 11.B1 cDNA library to find candidates. This is a reasonable first approach, but use of "normal" breast cDNA libraries would be better. We have obtained nearly normal cell lines and will incorporate use of those libraries in future studies.

Section 9: **Suppression of metastasis in human breast carcinoma MDA-MB-435 cells following transfection with the metastasis-suppressor gene, KiSS-1 [*Cancer Research* (1997) 57:2284-2287]**

Summary of major findings

In studies of melanoma, we identified a metastasis-suppressor gene called KiSS-1. The map location

of this gene at 1q32-q41 corresponded to a region often modified in late-stage human breast cancer. Therefore, we decided to assess whether KiSS-1 could suppress MDA-MB-435 metastasis. It does. The manuscript text is shown below.

Abstract

Based upon the observation that chromosome 1q deletions are not infrequent in late-stage human breast carcinomas, we tested whether the recently discovered human melanoma metastasis-suppressor gene, *KiSS-1* which maps to chromosome 1q32-q41, could suppress metastasis of the human breast carcinoma cell line MDA-MB-435. Parental, vector-only transfectants and *KiSS-1* transfectant clones were injected into the mammary fat pads of athymic nude mice and assessed for tumor growth and spontaneous metastasis to regional lymph nodes and lungs. Expression of *KiSS-1* reduced metastatic potential by 95% compared to control cells but did not suppress tumorigenicity. Metastasis suppression correlated with a decreased clonogenicity in soft (0.3%) and hard (0.9%) agar. While the overall rate of cell adhesion to extracellular matrix components was unaffected, *KiSS-1* transfectants spread on immobilized type-IV collagen more rapidly than control populations. Invasion and motility were unaffected by *KiSS-1*. Based upon the predicted structure of the KiSS-1 protein, our results imply a mechanism whereby KiSS-1 regulates events downstream of cell-matrix adhesion, perhaps involving cytoskeletal reorganization. In addition to its already described role in melanoma, our results show that *KiSS-1* also functions as a metastasis-suppressor gene in at least some human breast cancers.

Introduction

KiSS-1 was identified as a human melanoma metastasis-suppressor gene using subtractive hybridization between the metastatic human melanoma cell line C8161 and nonmetastatic variants generated following microcell-mediated transfer of chromosome 6 into C8161 (Lee et al., 1996). Transfection of *KiSS-1* into metastatic human melanoma cell lines, C8161 and MelJuSo, suppressed metastasis in athymic nude mice by 50-95% (Lee et al., 1996; Lee and Welch, 1997). The *KiSS-1* gene maps to chromosome 1 bands q32-q41, and predicts a hydrophilic, 164 amino acid protein with a proline-rich domain suggestive of an SH3 ligand and a putative protein kinase C-α phosphorylation site. These domains within KiSS-1 suggest that KiSS-1 could be involved in signal transduction pathway.

The purpose of this study was to determine whether *KiSS-1* might function as a metastasis suppressor in another tumor type. Breast cancer is the most common cancer among women in North America and western Europe, and is the second leading cause of female cancer deaths in the United States (American Cancer Society, 1997). Like most solid tumors, metastatic disease rather than the primary tumor itself is responsible for death. The rationale for studying *KiSS-1* in human breast cancer is based upon studies showing loss of heterozygosity for regions of chromosome 1q (Van de Vijver and Nusse, 1991; Gray et al., 1994; Munn et al., 1995). Involvement of chromosome 1q is not frequent in most cancers; therefore, the frequency of loss of heterozygosity was suggestive that a gene involved in breast cancer progression was encoded therein.

Here, we report a test of that hypothesis using transfection of the metastatic human breast carcinoma cell line MDA-MB-435 with *KiSS-1* cDNA. Transfectants formed significantly fewer pulmonary and regional lymph node metastases than control or neo-transfected clones. These results suggest that *KiSS-1* can function as a breast cancer metastasis-suppressor gene and may indeed be involved in the progression of human breast cancer toward malignancy.

Materials and methods

Cell lines and Culture

MDA-MB-435 and MDA-MB-231 are estrogen receptor- and progesterone receptor- negative, metastatic, ductal breast carcinoma cell lines (Price et al., 1990). Both cell lines form tumors when injected into the mammary fat pads of nude mice, and macroscopic metastases to lungs and regional

lymph nodes can be identified 10-18 weeks post-inoculation. However, MDA-MB-435 forms more metastases in a greater percentage of athymic nude mice than the MDA-MB-231 cells. Therefore, the former were chosen for transfection studies. MDA-MB-435 cells were the generous gift of Dr. Janet Price (U.T.-M.D. Anderson Cancer Center). MDA-MB-231 cultures were kindly provided by Dr. Robert Gillies (University of Arizona Cancer Center).

Both human breast cancer cell lines were maintained in DME-F12 medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (cDME-F12, Atlanta Biologicals) and no antibiotics. The neomycin-resistant *KiSS-1* transfectants were maintained in cDME-F12 medium containing 500 μg/ml of G418 (GIBCO-BRL, Gaithersburg, MD). All cell lines were free of *Mycoplasma spp.* contamination as determined by polymerase chain reaction-based test (PanVera, Madison, WI).

Transfection of KiSS-1

The construction of pcDNA3-*KiSS-1* expression vector was described previously (Lee et al., 1996). pcDNA3-*KiSS-1* vector was transfected into MDA-MB-435 cells using Lipofectin (GIBCO-BRL) according manufacturer's instructions. pcDNA3 vector only was also transfected as a control. G418-resistant clones were isolated by growth in selective medium. Uncloned, stable transfectant cells (MDA-MB-435 *KiSS-1* mix) were cloned by limiting dilution (MDA-MB-435-*KiSS-1* cl.1; MDA-MB-435-*KiSS-1* cl.2; MDA-MB-435-*KiSS-1* cl.3 and MDA-MB-435-*KiSS-1* cl.7).

Northern Blotting

RNA expression analysis was done as previously described. Total RNA from cell cultures and tumor tissues were isolated using a RNeasy Kit® (Qiagen, Chatsworth, CA). Poly(A)$^+$-enriched mRNA was isolated using Micro FastTrack kit® (Invitrogen, San Diego, CA). Total RNA (20 μg) or poly(A)$^+$ enriched RNA (2.5 μg) was size-fractionated in a 1% agarose gel containing 2.2 M formaldehyde. Following transfer and UV cross-linking, the nylon membranes were probed with a full-length ^{32}P-labeled *KiSS-1* cDNA.

Migration and adhesion assays

Migration was measured by the wounding method as described (Walther and Denhardt, 1996). Briefly, breast carcinoma cells (4×10^5) were plated onto 6-well plates in triplicate and allowed to grow until 80% confluence. The monolayer was scraped with a Teflon-coated plastic cell scraper (Fisher Scientific, Pittsburgh, PA) and the detached cells were removed by washing 3X with calcium- and magnesium-free Dulbecco's phosphate buffered saline (CMF-DPBS). The remaining adherent cells were incubated in DME-F12 medium containing 0.5% fetal bovine serum for 24 to 48 h. Photographs of the edge were used to compare migration of cells into the exfoliated space.

Adhesion was measured in a 96-well plate assay. Briefly, wells were coated with mouse laminin, fibronectin, or collagen type IV (Collaborative Biomedical Product, Bedford, MA) at the concentration of 5 μg/cm^2 for 1 h at room temperature. Murine adhesion molecules were used because this is the environment onto which cells would have to adhere *in vivo*. The wells were then rinsed with CMF-DPBS before pre-blocking with a solution of DME-F12 containing 1% BSA for 1 h at 37°C. After removing the supernatant, cells (4×10^4) suspended in adhesion medium (DME-F12 supplemented with 0.5 % BSA and 25 μM HEPES) were dispensed into each well, incubated at 37°C for varying times, and gently washed 3X with CMF-DPBS. Remaining adherent cells were quantified by measuring acid-phosphatase (Yang et al., 1996). In order to semi-quantify the spreading results, the percentage of spread/flattened cells per random high power field were determined.

Photographs of cells at various times after placement onto the matrices were done to compare spreading. Tissue culture plates (24-well) were coated with 5 μg/cm^2 mouse laminin, fibronectin, or collagen type IV as above. Cells (2×10^5) were suspended in adhesion medium and dispensed into each well before incubation at 37°C for 3 h. Unattached cells were removed by washing with CMF-DPBS.

Clonogenic soft-agar assays

Colonization in soft-agar was performed as described (Li et al., 1989) with minor modifications. A layer of cDME-F12 medium with 0.5% agarose was set into 6-well plates. A suspension of cells (10^2-10^3) in 0.3% agarose was overlaid onto the basal layer. A similar experiment was performed using hard agar (0.9%) as an upper layer since this parameters has been shown to be more closely related to metastatic efficiency than soft agar cloning (Li et al., 1989). The number of colonies was determined 20-40 days after plating.

Metastasis Assays

Cells (10^6) were injected into the subaxillary mammary fat pads of four- to six-week-old female athymic nude mice (7-10 mice per group, Harlan Sprague Dawley, Madison, WI). Mice were maintained under the guidelines of the National Institute of Health and The Pennsylvania State University College of Medicine. All protocols were approved and monitored by the Institutional Animal Care and Use Committee. Food and water were provided *ad libitum*. When mean tumor diameter (square root of the product of orthogonal measurements) reached 1.0-1.3 cm, primary tumors were surgically removed under Ketaset-Rompun anesthetic. Mice were then maintained for an additional four weeks to allow further growth of lung metastases. Following euthanasia, all organs were checked for metastasis. Visible lung metastasis were counted in fixed tissues (neutral-buffered formalin: Bouin's fixative 5:1) with the aid of dissecting microscope as described (Welch, 1997).

Statistical Analysis

The number of lung metastases in *KiSS-1* transfectants and control cells (parental and vector-only transfectants) was compared using a Kruskal-Wallis ANOVA of ranks procedure. The percentage of spread/flattened cells on type-IV collagen matrix was compared using Kruskal-Wallis ANOVA of ranks procedure. All calculations were performed using SigmaStat statistical analysis software (Jandel Scientific, San Rafael, CA). Statistical significance was defined $p \leq 0.05$.

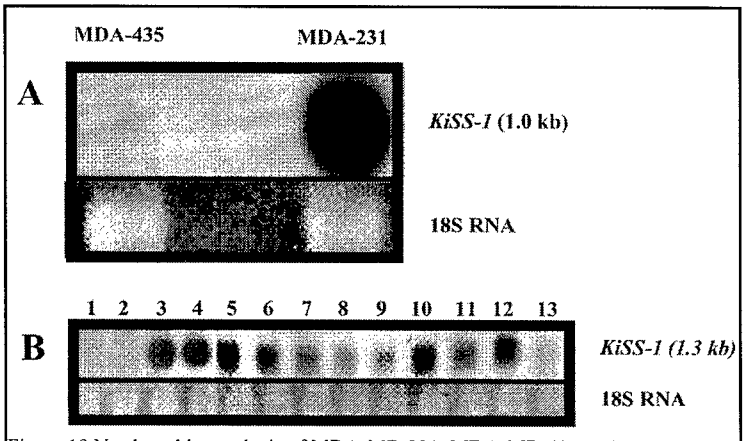

Figure 19 Northern blot analysis of MDA-MB-231, MDA-MB-435 and KiSS-1 transfectants for KiSS-1 mRNA expression. A. KiSS-1 expression in human breast carcinoma cell lines, MDA-MB-231 and MDA-MB-435. Poly(A)$^+$ enriched RNA was prepared from exponentially growing MDA-MB-231 and MDA-MB-435 cells. Agarose-formaldehyde gels were loaded with 2.5 µg RNA per lane, hybridized with radiolabeled full-length KiSS-1 probe. 18S RNA is shown as a loading control after the gel was stained with ethidium bromide. B. KiSS-1 expression in KiSS-1 transfected MDA-MB-435 cell lines. Total RNA was prepared from cultured cells and tumor tissue. Gels were loaded with 20 µg/lane, transferred to nylon membranes, hybridized with radiolabeled full-length KiSS-1 probe. 18S RNA is shown as a loading control after the gel was stained with ethidium bromide. Lane 1, MDA-MB-435 tumor; Lane 2, MDA-MB-435 vector-only transfectant tumor; Lane 3, MDA-MB-435-KiSS-1 mix cells; Lane 4, MDA-MB-435-KiSS-1 cl.2 cells; Lane 5, MDA-MB-435-KiSS-1 mix cells; Lane 6, MDA-MB-435-KiSS-1 cl.1 cells; Lane 7, MDA-MB-435-KiSS-1 cl.1 tumor; Lane 8, MDA-MB-435-KiSS-1 cl.2 cells; Lane 9, MDA-MB-435-KiSS-1 cl.2 tumor; Lane 10, MDA-MB-435-KiSS-1 cl.3 cells; Lane 11, MDA-MB-435-KiSS-1 cl.3 tumor; Lane 12, MDA-MB-435-KiSS-1 cl.7 cells; Lane 13, MDA-MB-435-KiSS-1 cl.7 tumor.

Results

Expression of *KiSS-1* transcript in MDA-MB-435 and MDA-MB-231 was measured by Northern blot (**Figure 19A**). MDA-MB-435 cells are reportedly less invasive *in vitro* than MDA-MB-231 cells, but are significantly more metastatic (Price et al., 1990). MDA-MB-435 cells do not express *KiSS-1* transcript (1.0 kb); but, MDA-MB-231 cells do. Based upon their relative *KiSS-1* expression and metastatic potentials, MDA-MB-435 cells were chosen for subsequent studies.

Full-length KiSS-1 cDNA was subcloned into the pcDNA3 constitutive expression vector and transfected into MDA-MB-435 cells. Concomitantly, vector without insert was transfected as a negative

control. Several single cell clones were randomly selected following limiting dilution, and expression of *KiSS-1* was confirmed by Northern blot (**Figure 19B**). Transfectants and matched controls were inoculated into the subaxillary mammary fad pads of four- to six-week old female, athymic nude mice. Tumors were measured weekly thereafter to assess growth rate. Once the mean tumor diameter reached ≈1 cm, tumors were surgically removed. One month later, mice were euthanized and metastases quantified. Primary tumor removal was done in order to minimize the effect of different growth rates for clonal populations, and to allow micro-metastases time to develop into macroscopic lesions. Local tumors were removed from MDA-MB-435, MDA-MB-435 *KiSS-1*mix, MDA-MB-435-*KiSS-1* cl.1; MDA-MB-435-*KiSS-1* cl.2; MDA-MB-435-*KiSS-1* cl.3 and MDA-MB-435-*KiSS-1* cl.7 injected mice 49, 49, 69, 106, 106, and 53 days after injection, respectively.

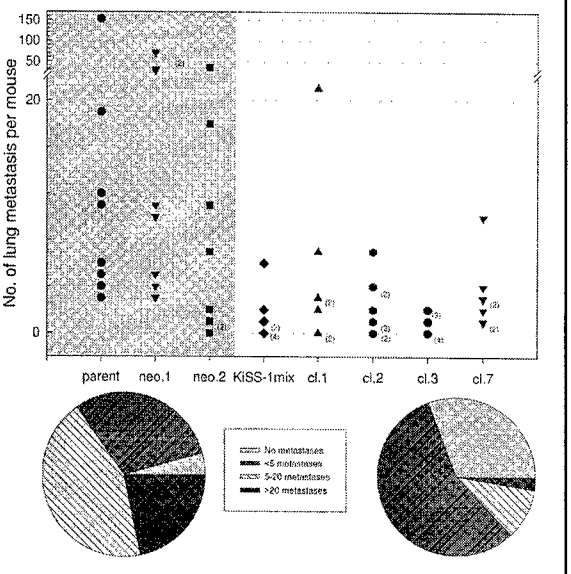

Figure 20 Formation of lung metastases transfectants is decreased in KiSS-1 transfectants. The upper panel depicts the number of metastases per mouse injected with variants of MDA-MB-435 (parent) cells. Each symbol represents one mouse. Numbers to the right of symbols indicate that more than one mouse had a particular number of metastases. The lower panel depicts the distribution of lung metastases number in control (shaded in upper panel) versus KiSS-1 transfectants. Cells (10^6) were injected into the mammary fat pads of 4- to 6-week-old female athymic nude mice. When mean tumor diameter reached ≈1.0 cm, primary tumors were removed and. The mice were maintained for four weeks to allow further growth of lung metastases.

Examination of hematoxylin- and eosin-stained sections of parental MDA-MB-435 and *KiSS-1* transfectant tumors was also done. Both tumor types exhibited characteristics of poorly differentiated, invasive adenocarcinoma (data not shown), but we found no consistent histologic properties that distinguished *KiSS-1* transfectants from parental cells.

Tumorigenic and metastatic properties are depicted in **Table 1** and **Figure 20.** All cells formed tumors; however, the number of macroscopic lung metastases in animals injected with the *KiSS-1* transfectants was significantly ($p<0.05$) decreased. *KiSS-1* transcript (1.3 kb) was still expressed in tumor tissues (**Figure 19B**), showing that tumor growth was not due to loss of the vector. Parental MDA-MB-435 cells formed an average of 26 lung metastases per mouse. Two vector-only transfectants formed 9 and 18 lung metastases, respectively. The *KiSS-1* transfectants formed between 1 and 5 lung metastases per mouse. These metastases were extremely small compared to those found in the parental cells or vector-only transfectants. Presence of additional microscopic metastases in random lung sections was not observed by hematoxylin and eosin staining (data not shown). Of parental and neo transfectants, only one mouse had no metastasis (1/24, 4.2%); whereas, 12/24 mice (50%) mice developed >10 lung metastases. In *KiSS-1* transfectants, 12/39 mice (30.8%) developed no lung metastases and only 2/39 mice (5.1%) had >10 metastases per animal. All *KiSS-1* transfectants were suppressed metastasis by at least 50 %. Likewise, the incidence of regional lymph node metastasis was reduced. These results clearly demonstrate that expression of *KiSS-1* in human MDA-MB-435 breast carcinoma cells significantly suppresses metastatic ability in athymic nude mice.

The mechanism by which *KiSS-1* suppresses metastasis in melanoma cells has still not been determined. Neither adhesion to extracellular matrices nor invasiveness *in vivo* or through reconstituted basement membranes using *in vitro* assays is altered in melanoma *KiSS-1* transfectants. These findings, however, could not preclude the action of *KiSS-1* at these steps in breast cancer metastasis. *KiSS-1* transfectants are as invasive as parental MDA-MB-435 cells *in vivo* (data not shown); therefore, *in vitro*

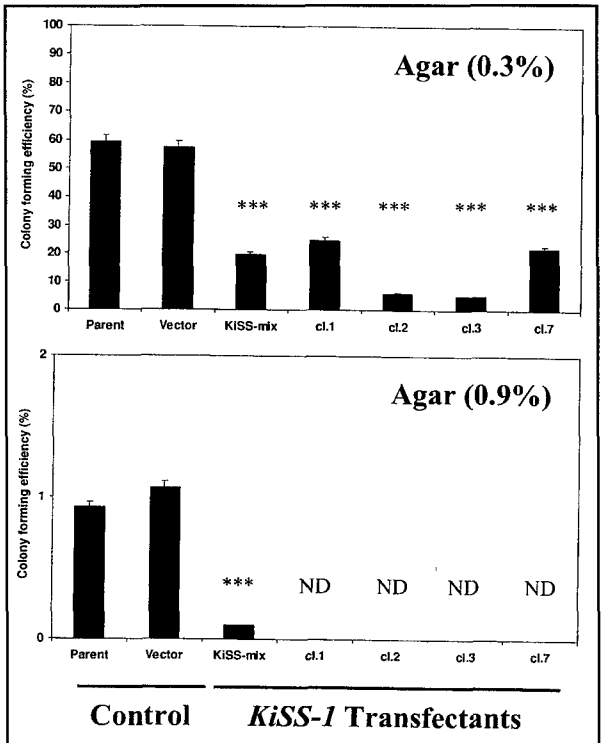

Figure 21. Clonogenicity of MDA-MB-435 variants in soft (0.3%) and hard (0.9%) agar. Values are mean plating efficiency ± S.E.M. KiSS-1 transfectants form significantly ($P<0.05$, ***) fewer colonies than parental or vector-only transfectants as determined by One-way ANOVA. ND, not tested.

assays were not done. Likewise, significant differences in migration were not observed (data not shown).

Although the kinetics of adhesion to tissue culture plates coated with murine collagen type IV, laminin, or fibronectin were unchanged in *KiSS-1* transfectants (i.e., the number of cells attached throughout the adhesion assay were identical to untransfected controls) (data not shown), the rate of spreading (i.e., flattened morphology) on type-IV collagen were different (**Figure 22**). Parental and vector-only transfectant cells were still predominantly rounded 3 hours after plating onto type IV collagen. Less than 5% of cells exhibited a flattened morphology. In contrast, more than 90% of *KiSS-1* transfectants has spread significantly in the same time.

KiSS-1 expression also correlated with a significantly reduced ability to form colonies in both soft (0.3 %) or hard (0.9%) agar (**Figure 21**). This parameter was examined because of published reports demonstrating a correlation between clonogenicity on hard and/or soft agar and metastatic propensity (Li et al., 1989).

Discussion

Based upon the relatively low frequency of chromosome 1q alterations in most human neoplasms except breast (Dracopoli and Bruns, 1994) and the map location of the recently described human melanoma metastasis-suppressor gene, *KiSS-1*, we hypothesized that restoration of expression of *KiSS-1* into a metastatic human breast carcinoma cell line should suppress metastasis. Our data clearly show that *KiSS-1* can function as a metastasis-suppressor in the MDA-MB-435 breast carcinoma. Parental MDA-MB-435 cells and tumor tissue did not express *KiSS-1* transcript. Tumor tissue collected from mice injected with *KiSS-1* transfectants continued to express *KiSS-1* mRNA. The lung metastases from *KiSS-1* transfectants were not visible prior to fixing with Bouin's and examination under a dissecting microscope; therefore, assessment of *KiSS-1* was not determined. Based upon past experience with these cells and this vector, the likelihood that the vector was spontaneously lost is low; but, this possibility cannot be excluded.

In preliminary experiments, *KiSS-1* transcript has been routinely detected in normal breast tissue adjoining biopsy specimens (data not shown). This suggests that *KiSS-1* may inversely correlate with progression of breast tumors. It will be necessary to extend our findings to a larger panel of cell lines or tumors with different metastatic potentials in order to establish firmly a role for *KiSS-1* in breast tumor progression. Unfortunately, there are few human breast carcinoma cell lines that reproducibly metastasize in athymic nude mice.

Although *KiSS-1* encodes a protein with a putative SH3 ligand, its mechanism of action remains unknown. SH3-domains are frequently found in many proteins that mediate protein-protein interactions important for signaling and cytoskeletal organization (Koch et al., 1991; Pawson and Gish, 1992). Thus, we have focused our attention on steps in the metastatic cascade with known signaling and/or cytoskeletal involvement.

KiSS-1 expression in melanoma does not correlate with decreased invasiveness nor ability to adhere

to basement membrane components. These conclusions were corroborated in the MDA-MB-435 breast carcinoma model. The ultimate levels of adhesion to extracellular matrix components did not change; however, *KiSS-1* transfectants did spread on type-IV collagen more quickly than control cells. The mechanism underlying this change is not known, but this is the first evidence that *KiSS-1* may be involved, at least tangentially, in cellular cytoskeletal organization.

Previous reports suggested that clonogenicity in agar, especially in hard agar, correlated with metastatic potential of MDA-MB-435 cells (Li et al., 1989). Our results generally agree with their findings. *KiSS-1* transfectants formed significantly fewer colonies than control cells. Again, this finding is consistent with altered cell structure in *KiSS-1* transfectants.

In summary, transfection of the human melanoma metastasis-suppressor *KiSS-1* into MDA-MB-435 human breast carcinoma cells significantly suppressed metastasis in athymic nude mice. This extends to three the number of cell lines transfected with *KiSS-1* that show marked suppression of metastasis without alteration of tumor forming ability. The results presented in this paper show that *KiSS-1* probably functions to control metastasis in a pathway shared by at least two tumor cell types of distinct embryonic origin — breast and melanoma. Our results also imply that *KiSS-1* might be a gene mapping to chromosome 1q that was previously implicated the breast cancer progression by loss of heterozygosity studies. And although they do not prove a role for *KiSS-1* in cytoskeletal organization, differential spreading on type-IV collagen and anchorage independent growth are consistent with the predicted function of *KiSS-1* as an SH3 ligand.

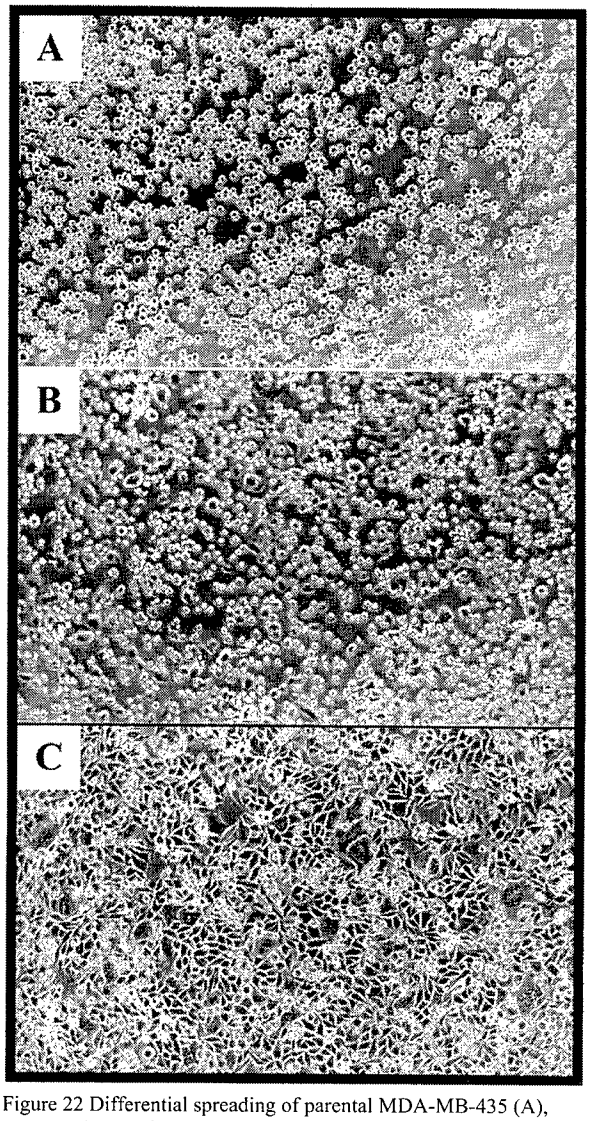

Figure 22 Differential spreading of parental MDA-MB-435 (A), vector-only transfectant (B) and KiSS-1-transfected (C) cells on immobilized type-IV collagen. Exponentially growing cells were plated onto type-IV collagen coated tissue culture plates and photographed 3 hours later. Note the increased number of spreading cells in panel C compared to panels A and B. Counting of spread/flattened cells within random high power fields was done to compare parental, neo-transfected and KiSS-1 transfected cells. Spread/flattened parental and neo-transfected cells comprise <5% of the population; whereas, >90% of KiSS-1 transfectants are spread three hours after seeding onto type-IV collagen matrix. Magnification, 100X.

Recommendations for follow-up experiments based upon these results

1. These results clearly show that KiSS-1 can function as a metastasis-suppressor gene in human breast carcinoma cells. As above, confirmation in an independently-derived cell line would be necessary to determine universality.

2. These experiments did not directly assess whether chromosome 11 induced KiSS-1 expression. A preliminary Northern blot revealed that some, but not all, of the neo11/MDA-MB-435 hybrids express KiSS-1. This raises the possibility that KiSS-1 may be a downstream mediator of the chromosome 11 genes' action.

PROGRESS AS RELATED TO STATEMENT OF WORK

This section is organized in the following way. The original statement of work is listed verbatim followed by a synopsis of progress.

Objective #1: Map the gene(s) responsible for suppressing metastasis of MDA-MB-435 to within 5 Mb by using MMCT with radiation-deletion variants of chromosome 11

Task 1-1 (Months 1-12): Identify polymorphic markers distinguishing MDA-MB-435 and donor chromosome 11

We have identified more than 20 polymorphic markers (Section 6)

Task 1-2 (Months 6-18): Prepare deletion variants of chromosome 11

Several chromosome 11 donors with deletions are in hand (Section 6)

Task 1-3 (Months 7-19): Prepare microcell hybrids with radiation deletion variants

This task has been initiated. Progress has been slower than expected.

Task 1-4 (Months 8-24): Confirm hybrids actually contain added chromosome 11 material by multiple criteria

This task has been initiated.

Task 1-5 (Months 12-24): Test hybrids for metastasis in orthotopic metastasis model

Task 1-6 (Months 24-48): Repeat above in independent series

Task 1-7 (Months 24-26): Map deletions in hybrids (1st set), prepare map of overlapping regions

Task 1-8 (Months 36-48): Map deletions in hybrids (2nd set), prepare map of overlapping regions

Objective #2: Stably introduce intact neo-tagged human chromosome 11 into MDA-MB-231 cells by MMCT

Task 2-1 (Months 1-6): Expand MDA-MB-231 cultures, verify pathogen-free (Mycoplasma free)

Completed

Task 2-2 (Months 6-12): Prepare chromosome 11 hybrids

Not done, see below

Task 2-3 (Months 10-18):Confirm hybrids actually contain added chromosome 11 material by multiple criteria

Task 2-4 (Months 8-24):Test hybrids for metastasis in orthotopic metastasis model

Task 2-5 (Months 12-24): Prepare chromosome 6 and chromosome 15 hybrids, repeat metastasis study

Task 2-6 (Months 24-36):Confirm hybrids actually contain added chromosome 11 material by multiple criteria

Task 2-7 (Months 24-36): Test hybrids for metastasis in orthotopic metastasis model

Task 2-1 was completed as expected. However, we decided that the MDA-MB-231 cells should be evaluated for metastatic potential prior to MMCT. They were not metastatic. Other potentially metastatic breast carcinoma isolates are being obtained. They will be tested as soon as possible.

Objective #3: Identify metastasis-associated genes in neo11/MDA-MB-435 cells using differential display and/or subtraction hybridization

Task 3-1 (Months 6-12): Prepare cDNA library from neo11/435.B1 cells, Prepare "screening" RNA blots

Completed

Task 3-2 (Months 6-9): Perform random primer amplification and repeat amplification for

differential display

Completed

Task 3-3 (Months 9-12): Perform "screening" Northern blots with probes from differential display

Completed two cycles

Task 3-4 (Months 12-18): Sequence positive sequences, determine novelty, obtain full-length

Completed two cycles

Task 3-5 (Months 18-24): Repeat Northern blots with longer probes for specificity

Completed two cycles

Task 3-6 (Months 9-18): Prepare subtraction library

Completed one RDA library

Task 3-7 (Months 18-30): Probe Northern blots with subtraction library

Task 3-8 (Months 36-48): Obtain full-length sequence for genes expressed in subtraction library

Progress has been as anticipated. The postdoctoral fellow overseeing this objective has been replaced by a technically and organizationally superior postdoctoral trainee. We hope that progress will be a little faster in the upcoming year.

Objective #4: Determine whether specific genes (such as KAI-1) is a metastasis-suppressor gene in MDA-MB-435 and MDA-MB-231 cells

Task 4-1 (Months 1-6): Prepare transfectants with KAI-1

Completed

Task 4-2 (Months 6-8): Select transfectants with increased KAI-1 expression

Completed

Task 4-3 (Months 9-18): Evaluate transfectants in orthotopic metastasis assay

Completed

Task 4-4 (Months 18-48): Prepare and evaluate transfectants prepared from genes isolated in Technical Objectives 1 and 3 above.

All four tasks have been completed and manuscripts submitted. This was made possible through collaborations with Drs. Barrett, Wei and Weissman, who had already initiated an alliance to study Kai-1 in breast cancer. Together, we made much faster progress than if we did the work alone.

CONCLUSIONS

Our preliminary data suggests that chromosome 11 encodes at least one human metastasis-suppressor gene. Our objective is to map and clone the gene(s) using parallel approaches. Approach 1 is to introduce pieces of chromosome 11, establish metastatic potential of the chromosome 11/breast cancer hybrids, map the gene(s) by regions of overlap. From July 1996-June 1997, we have established several baseline parameters necessary for completing this aim (i.e., identification of 19 polymorphic markers that discriminate MDA-MB-435 and donor chromosome 11) and availability of characterized chromosome 11 donors with defined deletions).

Approach 2 is to identify differentially expressed genes by differential display and/or representational differential analysis. Some candidate genes were identified, but most of the early candidates were apparently artefacts associated with the method used to generate the chromosome 11 donors. The methodology has been modified to minimize these false positive results. A third round of screening has been initiated and preliminary results are encouraging.

Candidate genes identified above were to be evaluated for their ability to suppress metastasis. Kai-1 was found to be more highly expressed in the neo11/MDA-MB-435 hybrids, suggesting that it might be

responsible for metastasis-suppression. A screen of human breast cell lines revealed that Kai-1 mRNA expression levels correlate inversely with tumor progression. Transfection studies also hint that Kai-1 may suppress metastasis; but, the answer is certainly not straightforward. Kai-1 is definitely not a particularly potent metastasis-suppressor gene in breast carcinoma.

Another objective of this grant was to determine whether our preliminary observations could be extended to other human breast cancer cells. Our plan was to introduce chromosome 11 by MMCT into MDA-MB-231. Unfortunately, the MDA-MB-231 cells were nonmetastatic. This was totally unexpected.

The bottom line is that we have made progress toward completion of all four specific aims. Aim 4 was completed faster than expected, but progress on Aims 1-3 was somewhat slower than anticipated. Nonetheless, we are continuing to make progress that approximates the time line proposed in the original proposal.

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Technical considerations for studying cancer metastasis *in vivo*

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The clinical problem of metastasis

At the time of diagnosis, tumors are already complex mixtures of cells despite having arisen from single cells. One of the earliest detectable changes in transformed cells is a greater genetic instability than their normal counterparts [1–8]. Even before they become tumorigenic, transformed cells (anchorage-independent, not contact-inhibited, and immortal) display genomic instability that is apparently the driving force for further progression. Genetic instability is particularly important for generation of heterogeneity within tumors [2,4,9–11]. A heterogenous population is then subjected to selective pressures that cause evolution toward increasingly malignant characteristics (i.e. invasion and metastasis). In addition to these types of intrinsic cellular changes, tumor cells exist in a complex milieu, exchanging signals with other tumor cell subpopulations and host cells. Heppner likens this to 'tumor cell societies' [12]. The sum of signals received by tumor cells, coupled with intrinsic properties, determines their individual behaviors as well as the overall tumor's behavior.

Metastasis is the ultimate step in the multistage process of tumor progression [13–21]. Subsets of neoplastic cells are able to form distinct tumor colonies from the primary tumor. Development of metastasis requires tumor cells to complete a complex cascade of events. First, malignant cells invade adjacent tissues and penetrate into the lymphatic and/or circulatory systems. Cells then detach from the primary tumor mass and disseminate. During transport, tumor cells can travel individually or as emboli composed of tumor cells (homotypic) or tumor cells and host cells (heterotypic). At the secondary site, tumor cells or emboli either arrest due to physical limitations (e.g. too large to traverse a capillary lumen) or bind to specific molecules in particular organs or tissues. Once there, tumor cells must then proliferate either in the vasculature or extravasate into surrounding tissue [22,23]. To form macroscopic metastases, cells must then recruit a vascular supply [19,24–31]. Fewer than 0.1% of cells that enter the vasculature survive to form clinically detectable macroscopic metastases [32]. At which step(s) of the metastatic cascade circulating tumor cells commonly succumb is debatable [22,23,33,34].

The greatest complication to successful cure of cancer is metastasis. During the past half-century, the proportion of patients surviving long-term has increased significantly [35]; however, patients diagnosed with metastatic disease invariably have poor prognoses. A majority die within 5 years. In contrast, when tumors are detected before they have spread, cure rates approach 100%. Unfortunately, 60–70% of patients have occult metastatic disease at the time of diagnosis [36]. Development of recurrence (whether local or distant) is also a bad sign. These statistics highlight how important it is to understand metastasis if treatment of patients with advanced disease is to improve.

Metastasis models

To understand metastasis, it is first important to recognize that tumor growth is a distinct process

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from development of metastasis. Not all cells within a tumor are able to metastasize (reviewed in [37]). Metastatic cells are a subset within a heterogeneous tumorigenic population that are endowed with additional capabilities to those required for uncontrolled growth. Therefore, to fully understand the metastatic cell, one must not only understand the steps involved in the conversion of cells from normal to neoplastic, but must superimpose the complexities of the many steps involved in metastatic cascade. It stands to reason, then, that if the objective is to understand metastasis, appropriate models designed expressly for this purpose will be required. Moreover, the appropriate use of these models is required. The purpose of this article is to highlight some of the idiosyncracies of metastasis assays and to identify potential pitfalls that can lead to spurious results and/or improper interpretation.

This paper was prompted by an incident at a recent meeting. One of my postdoctoral fellows had developed a series of cell clones from a human malignant melanoma. One clone was particularly metastatic; so, shortly after publishing its description, several investigators requested it from us. One of them approached us at that meeting and said, 'Those cells you sent us don't metastasize.' My postdoc was horrified at the thought that her results could not be reproduced. We knew that the cells had behaved as expected in replicate experiments in our lab as well as elsewhere. Nonetheless, upon our return, we immediately retested the cells *in vivo*. Fortunately, they behaved as expected. Subsequently, we found that the other investigator had slightly modified the conditions for growing the cells, maintaining the animals, length of assay and methods for quantifying metastases. 'Trivial' experimental parameters were altered by the other investigator with a catastrophic result. The investigator has since repeated the *in vivo* study according to our protocols and replicated our results.

No other incident in the laboratory better illustrated the need to reproduce experiments *exactly* before changing a single parameter. This incident also highlighted how little we actually know about the many variables in metastasis research. Further, it exposed the hazards of assuming that those desiring to study metastasis understand the complexities involved. In this vein, I decided to compile a list of conditions that are critical to consider when planning or executing metastasis-related studies. This had been done by Josh Fidler in a chapter published in 1978 [38]. At that time, Fidler wrote, 'Too many times investigators cannot reproduce published results because of their failure to conform to ***exact*** [emphasis added] experimental conditions.' He then added that, 'Sometimes ... subtle and totally unsuspected factors may profoundly influence the outcome of otherwise well-planned, well-executed experiments.' Some things have not changed. Some details have been discovered in the interim and an updated review was needed. As I began this project, I was reminded of his exhortations to study the history of our field. In doing so, many mistakes could potentially be avoided. Or as Santayana put it, 'Those who cannot remember the past are condemned to repeat it.' [39]

I hope that this attempt to summarize and to evaluate techniques employed for *in vivo* metastasis studies is useful for new and established investigators, just as Fidler's paper was for me early in my career. For the most part, I am confining this presentation to published results. However, some anecdotal accounts are presented because they are illustrative and because at least two seasoned investigators have described the same thing to me. As always, without critical review, anecdotal reports should be viewed with caution.

General considerations

Are *in vivo* studies required? Animal studies are costly, take a long time and are increasingly subject to regulation. *In vitro* assays are superior when eliminating variables and decreasing intra-experimental variability. However, no *in vitro* assay is fully predictive of a cell's behavior *in vivo*. As such, *in vivo* assays are the 'gold standard' for testing malignant behavior because *in vitro* models are of insufficient complexity to study the entire metastatic process: metastasis is not equivalent to invasion, adhesion, growth rate or susceptibility to immune cell killing. The phenotypes may correlate, but they are not equivalent. Any correlation established *in vitro*, must be verified using properly designed *in vivo* studies.

When designing models for studying cancer metastasis, two criteria must be met. The first should be obvious: use metastatic cells (the 'seed'). Unfortunately, some investigators have used non-metastatic cells for their studies. Some rationalize that if cells were derived from metastases, they are *de facto* metastatic. This conclusion does not necessarily follow. Virtually all human breast cancer cell lines were isolated from metastases or pleural effusions; however, few (e.g. MDA-MB-435 and MDA-MB-231) reproducibly form macroscopic metastases in a majority of immunocompromised mice [40]. Additionally, some studies started with metastatic

cells, but the conditions under which the cells have been maintained have rendered them nonmetastatic. Regardless of whether inherently nonmetastatic cells are used or whether the cells have lost metastatic potential, they are essentially useless for studying metastasis.

The second criterion for metastasis assays is that the tumor cells must grow in relevant organ environments (the 'soil'). Issues related to the soil will be elaborated below. Suffice it to say, the outcome of any metastasis study depends upon both tumor cell and host cell properties. Since metastasis assays require the use of animal models, the issue of 'soil' is not trivial. The first section reviews those parameters associated with the use of experimental animals. This is followed by sections describing conditions affecting cell preparation for metastasis assays.

Animal models of cancer metastasis

What defines an appropriate animal model? The details are controversial. Key considerations are: What hypothesis am I testing? and Will this model allow me to test that hypothesis? A model is appropriate as long as it provides controlled conditions in which to test a given hypothesis. Of course, any 'model', by definition, is imperfect. The key is to recognize the strengths and limitations of a model and then to work within those parameters. At what point does a model become inadequate? If the question being addressed or the hypothesis being tested cannot be unambiguously tested using a particular model, it is necessary to improve the model or switch to another. As importantly, it is crucial not to pose a hypothesis on the basis of the model. Rather the model should be chosen because it allows testing of the hypothesis!

Early experimental studies of metastasis utilized inbred, but not syngeneic strains [41]. Therefore, some types of studies (e.g. immunological control of metastasis) should be viewed with caution. However limited, these early studies set the groundwork for modern metastasis research. Indeed, many of these models still provide useful information [42]. A major advance was the development of syngeneic tumor cell lines and animal hosts (reviewed in [43–47]). In particular, selection of sublines of the B16 murine melanoma ushered a new era for metastasis research [48]. Numerous additional syngeneic models of metastasis were developed and characterized. A great deal of fundamental understanding of the metastasis phenotype was achieved using this and similar models. However, even these models were limited because the cell lines being tested were nonhuman (typically rodent, although feline [49], avian [50], amphibian [51] and canine [49] models also exist). As a result, extrapolation to human disease was imperfect.

The development and widespread availability of immunocompromised hosts opened the door for studies of human cancer metastasis. In particular, athymic nude mice [44–47,52–54] and subsequently SCID (severe combined immunodeficiency [55,56]) mice have been widely used. Additionally other immunocompromised hosts, for example the chick embryo, have been used [57,58]. Xenografts (i.e. human cancer cells grafted or inoculated into rodents) have now become more commonly studied than animal tumors in many labs. Even though human cells are being used, studies in immunocompromised hosts are still limited by the rodent background. When metastases develop, they do not always display the patterns of distribution seen in human cancer [14,20,59–64]. These discrepancies are still not understood.

Model improvements and refinements continue to occur. Yet, the limitations of each model must be recognized and addressed when designing and interpreting *in vivo* experiments. Regardless of host strain, all *in vivo* animal models share critical parameters. Rather than an exhaustive review of individual models, subsequent sections will focus on rodent models. Similar considerations apply when using other species.

Choice of species/strain

Ultimately, the decision of which research animal host to use is based upon the metastatic cells being evaluated. This decision is initially dependent upon immunologic considerations. Whenever possible, syngeneic animals should be used. This most closely recapitulates tumor development and progression. Even more relevant are autochthonous models [65]. The major limitation of the latter is the large number of animals required for statistical analysis. The use of transgenic animals may overcome this limitation [66]. When autochthonous and syngeneic animals are not available, related haplotypes should be employed to reduce the impact of allograft or xenograft rejection.

Analysis of tumor growth and metastasis of human cancers requires the use of immunodeficient animals. The most common animal host for xenotransplantation is the athymic nude mouse. Xenografts generally retain morphologic and biochemical characteristics following transplantation

[47,54]. For some tumor types (e.g. melanoma, soft tissue carcinomas and sarcomas), 'take' rate of fresh tumor specimens is acceptable; but for others (e.g. breast [67], stomach and prostate [68]) results are unacceptably low [69]. Some early studies showed low incidence of metastasis of human tumors in nude mice [44,53], which was interpreted by some to demonstrate the poor utility of nude mice as an appropriate model for metastasis. However, appropriate experimental design (namely the site of injection, see below) was not fully understood and therefore not uniformly used. Likewise, optimal conditions for maintaining viability and minimizing artificial selection of tumor explants have still not been determined.

More recently, SCID (*xid*) and beige (*bg*) mice have become more readily available and less expensive. In addition to the T-cell deficiency of athymic nude mice (*nu/nu*), SCID mice also have B-cell deficiency [70]. Mice with the *bg* mutation are deficient in natural killer (NK) cells. And there are mice with triple deficiencies. Intuitively, one would predict that metastatic potential would increase as mice become more immunodeficient. This is not always the case [55,71–74]. For example, using the MDA-MB-435 human breast carcinoma cell line, incidence (no. of mice with metastases/no. of mice injected) and quantity of metastasis (no. of metastases/mouse) in SCID [15/15; median 5] mice were greater in NCr nude mice [8/14; median 21] which, in turn, were surprisingly greater than in triple-deficient NIH-3 mice [1/15; median 0] [40]. Likewise, Garafalo *et al.* systematically compared tumor growth and metastatic potential in athymic nude, beige, beige–nude and SCID mice. Metastatic potentials in different hosts were cell line-dependent [72]. Tumorigenicity, metastatic potential (incidence of metastases) and patterns of metastasis (location of secondary lesions) were all variable. Rank order of metastatic potentials was similar (i.e. poorly metastatic cells in one host were generally less metastatic than other cells in different hosts and vice versa); however, one cannot presume that more complete immunosuppression will result in a corresponding increase in tumor 'take' and/or metastatic potential.

Animal health

Overall animal health, although easily controlled, is often ignored when performing *in vivo* tumor studies. Animals with bacterial, viral and parasitic infections can profoundly impact experimental outcome, presumably due to alterations of the host animal's immune system. Animals infected with endoparasites (pinworm, tapeworm, etc.), ectoparasites (lice, mites, etc.), viruses (minute mouse virus, mouse hepatitis virus, hepatitis, pneumonia virus, etc.), bacteria (*Pseudomonas*, *Staphylococcus*, etc.) or *Mycoplasma* often do not respond to tumor challenge identically to healthy animals.

Relationships between host homeostasis, immunity, tumor growth and metastasis are still not well understood even though significant inroads have been made. These topics are beyond the scope of this review. Readers are referred to excellent reviews [75,76]. However, it is obvious that immune response is dependent upon both tumor cell antigens and the host animal's ability to mount an immune response to those epitopes.

Hyperchlorinated drinking water (12–16 ppm [77]) was first given to experimental animals to avoid radiation-induced death resulting from opportunistic pathogenic enteric bacteria (e.g. *Pseudomonas*). The practice became more widespread in an effort to minimize the possibility of contamination within animal breeding colonies. With time, a 'more is better' philosophy began to prevail and higher chlorination levels (up to 30 ppm) became commonplace. Unfortunately, at these higher levels, peritoneal exudate cellularity and animal growth (i.e. weight gain) dropped dramatically, suggesting that macrophage hematopoiesis and/or activity may have been compromised in a dose-dependent manner [78]. Since macrophage immunity can influence efficiency of metastasis [79,80], husbandry conditions must be monitored carefully. Chlorine is not unsafe; however, levels should not be above 10–15 ppm. In some geographic regions (particularly those with supplies from surface water sources, like rivers or streams), tap water is heavily chlorinated; therefore, it is incumbent upon the investigator to routinely monitor chlorination levels.

Kyriazis *et al.* [81] showed that the proportion of mice developing tumors was lower in mouse hepatitis virus (MHV)-infected mice compared to healthy mice. Kameya [82] also reported that the percentage of human choriocarcinomas that could be established in specific pathogen-free nude mice was higher than in mice housed in conventional facilities. Together these highlight the importance of the animal facility in studies of cancer biology. Many animals may have subclinical infections (i.e. not detectable without serologic or histologic testing). These infections are insidious because everything appears normal when, in fact, animals are sick. Depending upon the length of an experiment, asymptomatic infections could be overlooked unless precautions are in place. An example from my

laboratory demonstrates clearly how this type of subclinical infection can impact results from metastasis assays.

Rats do not become infected by MHV [83]. However, leukocyte counts can become elevated, sometimes significantly, if MHV is in the animal facility (Note: not necessarily in the same room). Thus, MHV stimulates the rat's immune system. Using the 13762NF rat mammary adenocarcinoma in syngeneic F344 rats housed adjacent to a mouse room infected with MHV, we found that metastatic potential was significantly lower (sometimes reduced by $>75\%$) than if no infection was present in the animal facilities. Once the MHV infection was eliminated, frozen aliquots of cells were retested and shown to have higher metastatic potentials. Similar results (i.e. decreased metastatic potential) were obtained in murine melanoma cells tested in MHV-infected, asymptomatic syngeneic mice. We suspect MHV-enhanced immune cell killing of circulating tumor cells; however, we could not exclude the impact of other opportunistic organisms or co-factors.

Please note that MHV is highly virulent, readily transmitted between rooms and difficult to eliminate without depopulating an entire rodent facility (not just the room in which the infection was found!) [83]. Serologic results from sentinel mice were obtained after the experiments had been completed, thereby allowing this interpretation. Thus, good record keeping allowed us to explain an unexpectedly low metastatic potential. If, however, previously untested cells were being evaluated, we may have mistakenly presumed low metastatic potential.

Reputable vendors now provide specific pathogen-free (SPF) or virus antibody-free (VAF) rodents. The slightly increased cost of these animals is justifiable in light of better control of experimental variables. However, the handling of animals in the colony must continue to maintain the disease-free, pathogen-free status. This is a labor-intensive undertaking; however, carelessness in animal handling will compromise results. This cannot be tolerated. Institutional Animal Care and Use Committees (IACUC) now require training in animal care for investigators and their technical staffs. Likewise, personnel who change bedding, water, etc. should be trained in aseptic technique in order to minimize cross-contamination. The importance of proper handling and maintenance of aseptic/sterile technique cannot be overemphasized. All infected facilities should be depopulated (completely) and re-established using only virus- and pathogen-free animals. While this can be costly, eliminating variables will provide more consistent, reproducible and more easily interpretable results. In relatively short order, cost savings due to more reliable results will overcome the initial outlay. If unique strains are already infected, they should be housed in completely independent, isolated facilities with handlers who do not come into contact with other research animals.

Immune status is also modulated by other mundane, but nonetheless important, parameters that are unrelated to infection. A detailed discussion of age-related immune changes is discussed below. Corticosteroid levels are high in mice immediately following transport. Corticosteroid levels can cause restriction of lung capillary lumen diameter which would increase nonspecific retention of tumor cells and/or emboli. This is a proposed explanation for enhanced lung colonization by Ehrlich ascites tumors in mice treated with hydrocortisone [84]. Corticosteroids also suppress a number of immune cell functions [85] which could influence metastatic potential. It is desirable to allow animals acclimation time to allow corticosteroid levels to return to normal. This normally takes a minimum of 48–96 h. Joy Ware (Medical College of Virginia) has found that tumorigenicity of the human prostatic carcinoma cell line, PC3, is even more susceptible to transport-induced changes. She found that tumorigenicity was only 50% if the mice had acclimated 1 week; but rose to 100% if 2 weeks had elapsed. Clearly, recovery from whatever occurred during transport required additional time if this model system was to be used.

Transport is only one parameter that can induce stress in research animals. Conditions within the animal facility should be consistent: 12 h light cycle, type of bedding, constant humidity and temperature. Extreme noise and improper handling techniques have been shown to alter stress levels in experimental animals [86]. Based upon the relationships between stress and immune cell function [84,87–89], it is not unreasonable to expect metastasis to be impacted by stress. However, detailed studies have not, to my knowledge, been undertaken. Some investigators have reported suspicions that construction projects causing loud noise near the animal facility may have impacted tumor behavior.

Metastases are more frequent at the site of tissue trauma than in healthy tissue. This is in addition to the hypothesis that surgery may result in a 'showering' of tumor cells and, in fact, cause metastasis in patients [90–92]. There is still some controversy regarding the latter hypothesis; however, Murthy and colleagues have developed animal models that

clearly demonstrate formation of metastatic lesions (distinct from local recurrence) at the site of well-defined surgical wounds [93–95]. Although all of the details have not yet been elucidated, growth at these sites is presumably due to the rich milieu of growth factors present at the site of wound healing [96]. This assessment of mechanism implies that wounds or tissue trauma in experimental animals should be minimized in order to avoid spurious results.

Evidence that cytokines released during inflammatory responses will influence metastatic cells is indirect, but compelling. Metastatic human melanoma cells express high levels of the adhesion molecule ICAM-1 [97,98]. Surface ICAM-1 expression appears to directly correlate with metastatic potential [98,99]. However, we found that the basal ICAM-1 expression in a panel of human melanoma cell lines was not always predictive of metastatic efficiency in nude mice [100]. In each melanoma cell line treated *ex vivo* with interleukin-1 or tumor necrosis factor-α, surface ICAM-1 levels rose with a corresponding increase in metastatic potential [100,101]. These cytokines also enhanced release of soluble ICAM which is thought to play a role in evasion from immune cell killing [102]. The precise mechanism (i.e. at which step in the metastatic cascade) by which metastatic potential is increased remains unknown.

Although caging, bedding and housing conditions are now regulated in most countries, some facilities do not strictly adhere to them. Readers are referred to Refs [103] and [104] for details. It is always safe to follow the guidelines established by the American Association for Accreditation of Laboratory Animal Care (AAALAC) for facilities and the American Association for Laboratory Animal Science (AALAS) for care and use issues. Recent evidence indicates that levels of exercise (partially a function of space) can influence tumor development in experimental animals [105,106]. To my knowledge, systematic studies comparing lethargic vs exercising mice for metastatic potential have not been done. Until then, these should be considered potentially important variables.

On a final note, it is the principal investigator's responsibility to ensure the health and well-being of the research animals. This is usually delegated to technicians who in turn delegate routine day-to-day monitoring to personnel untrained in the nuances of the research. While understandable in terms of cost, valuable resources are being wasted. Personnel who handle the animals daily are more likely to observe subtle changes in behavior or health. It is a good idea to thoroughly brief the animal care technicians regarding the nature of the experiment and the expected results. This small investment in time not only increases their 'buy-in' to the experimental process, but also multiplies the number of people monitoring an experiment with a critical eye. Catching changes early can alleviate an epidemic. Moreover, observing subtle behavioral changes (e.g. change in gait, tendency to turn in circles, etc.) may be useful in identifying rare brain metastases.

Animal source

In addition to providing healthy, uninfected animals, vendors design breeding programs to minimize drift and routinely test their breeding stocks in order to assure that the strains' genotypes have been maintained with fidelity. None of the key histocompatibility or genetic markers will have changed; however, this does not preclude other evolutionary processes taking place. It may be worthwhile for some investigators to compare side-by-side metastasis results from animals from different vendors supplying the same strain.

Although genetic drift does not appear to be a major problem, some investigators who have used a particular strain of cells for extended periods suspect that the animal strains have changed over time. Their suspicions are based upon repeated experiments over several years using early passage frozen cell stocks. Metastatic potentials for some cell lines have steadily decreased. Whether other variables that changed (e.g. serum used for cell cultures, etc.) could account for some of the differences in behavior cannot be completely discounted. Presently, there is no way to control for this variable except to use the most reputable vendors, who utilize specific breeding protocols to minimize drift.

Caution should also be exercised even when the same strain is purchased from different vendors [107]. While all of the animals are derived from founder parents, those in different breeding colonies are considered different strains after only a few generations [103].

Animal gender

The gender of host animals can profoundly affect metastasis, especially in hormone-responsive tumor cells. It is logical to study breast, endometrial and ovarian tumors using female mice, and prostate and testicular tumors using male mice. But this is not always done. There are several cases where hormonal status of a host animal has influenced

metastatic behavior in mice and rats [108–110]. In addition to obvious reasons for gender selection, female mice are often chosen because they tend to fight less than males. Fighting among males is less, however, if there are no females in the room, thereby eliminating the stimuli to prove their dominance and virility. This issue can be particularly important for surface tumors that might be affected by cage skirmishes.

For non-hormone-dependent tumor types, like lung cancer and melanoma, gender would not be expected to profoundly impact metastatic potential. However, there have been some surprises. B16 melanoma cells grew more slowly in syngeneic female C57BL/6 mice than in syngeneic male mice. This difference was abolished when females were oophorectomized [111]. Ehrlich ascites cells metastasized more in female CFA mice than in males [112]. Thompson [113] showed that mammary adenocarcinomas produced more metastases in female than in male mice. And it has been observed that G6 tumors metastasize more to lung in pregnant mice than in virgin mice [114]. The most common explanation for differences in metastatic potential in these experiments is hormones, although definitive proof is required. Supporting this notion that hormones are involved, are recent findings that metastasis-associated phenotypes, like expression of tissue inhibitors of metalloproteinases, can be transcriptionally regulated by hormone receptors [115].

Age

Establishment of metastasis is dependent upon the ability of tumor cells to attach to and traverse the intimal layers of vessels. With age, multiple parameters associated with the vessel architecture and hemodynamics are altered, e.g. endothelial integrity, atherosclerosis, vessel diameter, inflammation, patterns of blood flow [116]. Such changes in hemodynamic properties would be expected to influence metastasis [117]. Several studies have shown that metastatic potential of simultaneously prepared tumor cells is different when the cells are injected into mice differing only in age at the time of injection [118,119]. In one study, the level of growth hormone was correlated with metastatic potential [120]. Whether the growth hormone was directly responsible for shifts in metastatic potential was not systematically evaluated.

Thompson [113] compared lung colonization following intravenous inoculation of tumor cells in 15- and 71-week-old mice. The number of lung colonies was greater in 'old' mice compared to the younger mice. This pattern is paradoxically opposite to that seen in one human cancer study. Erschler evaluated autopsy data from over 10 000 cancer patients and found that incidence of metastasis was greater in younger people than in older people [121]. In patients ranging in age from 20 to 30 years, incidence of metastasis was 80% in contrast to < 50% for patients over 70 years old. Regardless of trend direction, both studies highlight age-related differences in metastatic potential.

Ware *et al.* also found that human prostatic carcinoma cell lines metastasized to different degrees, depending upon the age of the athymic nude mice [122]. Metastatic potential also varied according to the site of injection.

One of the best described age-related changes in mice is natural killer (NK) cell activation. Hanna and colleagues found an inverse correlation between NK activity and incidence of metastases in murine tumors [123]. Definitive roles for NK cells in metastasis were established when NK cells were depleted by treatment with cyclophosphamide [124], β-estradiol [125], anti-NK antibodies [126] or anti-asialoGM1 [127]. They further suggested that high NK levels in athymic nude mice might be responsible for the generally low metastatic efficiency of human tumors [123,128]. This hypothesis was supported by experiments comparing two strains of nude mice with markedly different NK levels. BALB/cAnN mice exhibit lower NK-cell-mediated cytotoxicity than identically housed age-matched N:NIH(S) mice. More metastases develop in BALB/cAN mice (3 weeks) than in age-matched N:NIH(S) mice [128].

Young mice (3 weeks old) have lower NK activity than older (6–8 weeks old) mice [126,129,130]. Fidler and colleagues injected tumor cells subcutaneously or intravenously into young and old mice. All mice developed subcutaneous tumors; however, metastases developed only in i.v. injected young mice [128,131]. This study underscores the importance of NK activity and age because, unlike tumor cells growing in a primary tumor mass, metastatic progenitors are circulating in the blood stream or lymphatics as single cells or small clumps. As such, they are more vulnerable to destruction by immune and nonimmune mechanisms. Therefore, the same cells can exhibit apparently different metastatic potentials in young vs old mice. As a general rule, it is recommended that mice as young as possible be used if metastasis is a desired end point [44].

Another example of age-related changes affecting tumor cell behavior has been described using

transformed rat liver epithelial cell lines [132]. In young rats, tumors formed following subcutaneous (s.c.) or intraperitoneal (i.p.) transplantation into syngeneic rats but no tumors were formed (or were weakly tumorigenic) following transplantation into livers of young adult rats. When injected into the livers of increasingly aged rats, tumorigenicity increased, however. These results suggested that the liver parenchyma changes with age and analogous changes in other organs may likely occur.

Other animal parameters

Metastasis formed by B16 melanoma variants was lower in genetically obese mice than in lean C57 background mice [133]. Besides the obvious implications relating obesity and malignant behavior, more indirect implications regarding diet and metastasis were inferred. Several studies showing modulation of metastatic potential in animals receiving different diets have been done [134–139]. Generally, rodent chows used in animal facilities are complete and balanced. However, it is important to be aware that tumor cell behavior could change if diet is modified. It will be interesting to see experiments evaluating metastasis in mice with the different genetic changes resulting in obesity [140,141].

Most investigators recognize the need to sterilize food prior to feeding immunocompromised animals. However, autoclaving destroys the nutritional makeup of the chow. Specialized autoclavable formulations are required. In addition, it is important to work closely with an animal facility because economics can force a change of chow sources (hence, formulation) within or between experiments. Minimum nutritional requirements are surely met; however, additives or proportions may vary considerably. It is always a good idea to be aware of changes in supplier. If results vary unexpectedly or are not reproducible, these variables can be systematically examined as needed.

Development of metastasis takes time. Depending upon the tumor cells being evaluated, metastases can develop shortly after injection/implantation or following prolonged periods. If metastases arise only after tumor burden is large, then multiple humane considerations deserve increasing consideration. No one wants to cause unnecessary suffering in the experimental animals. Besides the ethical considerations, large tumor burdens can complicate experiments. It is phenomenal how well some mice accommodate extremely large tumor burdens. Overall well-being appears unaffected, even when mean tumor diameter exceeds 2 cm. Once tumor burden begins to compromise animal weight, feeding patterns, mobility, behavior or coat texture, the experiments must be terminated immediately. Therefore, it is necessary that monitoring become more frequent during the course of *in vivo* experiments. By doing this, premature termination of experiments solely on the basis of tumor size will be less likely. By closely monitoring the animals over a longer period, the likelihood of finding metastases increases.

Site of injection: 'experimental' and 'spontaneous' metastasis assays

Animal models for metastasis most commonly involve two approaches. The first involves inoculation of tumor cells into tissue sites (i.e. subcutaneously, intradermally, intramuscularly, or into specific organs or tissues) which results in the formation of a local tumor that will eventually give rise to spontaneous metastases. The second approach circumvents the steps of local tumor growth and intravasation by directly introducing (usually intravenously, but also intraarterially or intracardially) tumor cells directly into the vasculature. This results in formation of experimental metastases. Both methodologies have contributed to our understanding of the multigenic, multistep metastatic phenotype; however, the experimental metastasis assay has been maligned by some [142–147].

The reasons for questioning the validity of the experimental assay are based on the following arguments. (1) Bolus injection of tumor cells does not accurately represent the continuous release of tumor cells from a primary tumor into the vasculature. However, the evidence argues that transient changes in hemodynamics and homeostasis can cause a 'showering' of tumor cells into the blood [148] and changes in blood flow patterns [149]. (2) Lack of prior immune presentation eliminates the possibility for development of an immune response. Experiments designed to address the issue of 'tumor-sensitization' suggest that immune killing of blood-borne tumor cells due to specific immune responses do not play a major role in metastatic potential [145,150–152]. Other data suggest that some locally growing tumors secrete factors that can enhance metastasis [153,154] or factors which modulate the function of immune components that, in turn, enhance metastatic efficiency [155,156]. Conversely, some tumor cells produce factors that inhibit the growth of other tumor cells and metastases [157–164]. Therefore, the assay may be flawed in

terms of cell–cell communication between the locally growing tumor and tumor cells present elsewhere. (3) Experimental metastasis omits the first steps of the metastatic cascade and may overstate metastatic efficiency. This is correct.

Indeed, spontaneous metastasis assays more closely mimic the metastatic cascade than the experimental metastasis assay: all steps subsequent to transformation are replicated. As a result, it is the superior method for studying the entire process of metastasis. However, the experimental metastasis assay has certain advantages over the spontaneous metastasis assay. First, since it bypasses some steps, the number of variables involved in the experiment is smaller. This leads to a significant reduction in statistical variability in most cases. Second, experimental metastasis focuses on specific steps in the metastatic cascade. This allows studies on the latter steps of the metastatic cascade without the complicating factors associated with primary tumor growth. Third, since tumor growth is not required and since cells are injected directly into the vasculature, the time required for the experiment is often significantly shorter. As a result, experimental costs are less. Fourth, complications (e.g. pain or suffering) due to large tumor masses are alleviated.

These points are not raised to advocate the experimental assay over the spontaneous assay. Rather, they are raised in order to identify considerations when designing experiments. Full characterization of any metastasis model system should include both assays. If results (i.e. incidence of metastasis and/or pattern of metastasis formation) are comparable, then either assay can be used. If, however, the results differ depending upon the method used, a limitation to a particular model has been identified and should be avoided. Conversely, such discrepancies provide the opportunity to identify specific biochemical or molecular reasons underlying the differences. Experimental metastasis correlates well with ability to spontaneously metastasize from a tissue site in some cases [71,164–167], but not others [40,53,144, 146,168,169]. Therefore, it is incumbent on each investigator to establish usefulness of each assay on a case-by-case basis.

Some argue that injection of thousands or millions of tumor cells directly into the vasculature does not reflect the situation in humans; however, quantification of tumor cells in patient blood indicates that these numbers are not unreasonable [170,171]. Indeed, mere presence of large numbers of tumor cells in the blood does not necessarily mean that macroscopic metastases will develop [170]. Perhaps a more relevant technical concern would be the condition of the cells at the time of injection (see below) and the site at which cells enter the vasculature. Although injection of tumor cells into the vasculature results in wide distribution of tumor cells throughout the body [172–174], the greatest number of cells interacting with any tissue will be at the first capillary bed encountered. Thus, the site of injection can enhance the likelihood of developing metastases in a particular organ. This strategy has been exploited in order to select for subpopulations of cells with increased propensity to colonize a particular organ. For example, injection into the carotid artery was used to select for brain metastasizing variants [175,176], and intrasplenic, intraportal and intrahepatic injections have been used to select for liver-colonizing variants [177–180], whereas, injection into the lateral tail vein commonly results in lung colonies [48,181]. The latter observation leads to a justifiable criticism of the typical experimental metastasis assay: inoculation of tumor cells into the lateral tail vein of mice with subsequent quantification of lung metastases. If lungs are not a common site of metastasis for that type of tumor, relevance is somewhat diminished. This criticism must be weighed against the other advantages of the model, however.

Orthotopic injections

A cursory review of the literature reveals that the most common site for injecting tumor cells when performing spontaneous metastasis assays has been the subcutis. The major reason for this is convenience: subcutaneous injections are easy and tumors readily visible for monitoring growth. But most progressively growing tumors fail to metastasize from this site [182]. Therefore, even if human tumors growing subcutaneously in nude mice maintain most of their morphological and most biochemical characteristics, it is questionable whether the subcutis is the most appropriate site for studying metastasis. The ideal *in vivo* environment for studying metastasis should mimic the autochthonous situation as closely as possible, i.e. orthotopic. Some investigators contend that orthotopic implantation is essential [18,183–187].

Kinsey first reported differential colonization efficiency of S91 Cloudman melanoma cells in mice depending upon injection site in 1961 [188]. Others have revisited the observation over subsequent years [136,167,169,174,184,189–204], but Fidler and colleagues [183,205–209] and Hoffman and colleagues [185,210–216] have raised awareness of the issue of orthotopic implantation to a higher level. Their data

clearly support the notion that relevant models require beginning with injection/implantation into a relevant injection site.

Orthotopic implantation often results in higher efficiency of metastasis and more relevant colonization patterns (i.e. similar to human cancer) than do simultaneously prepared ectopic implantations into age- and sex-matched mice. This finding has been demonstrated for colorectal tumor cells injected into the caecum [177,206,217–223], renal carcinoma cells injected into the kidney or under the subrenal capsule [205,224], cutaneous melanoma cells inoculated intradermally [197,200,225–227], ocular melanomas implanted into the eye [190,216], bladder carcinomas placed into the bladder wall [201,228,229], breast carcinomas injected into mammary fat pads [136, 196,199,230–232], prostatic carcinomas injected into prostate [208,233], pancreatic carcinomas inoculated into pancreas [215,234–238], osteosarcomas injected into bone [167], neuroblastoma implants in the adrenal gland [198,239,240], gastric adenocarcinomas injected into the stomach wall [241], and lung tumors implanted into the bronchi [203,242].

Using a modification of orthotopic injection directly into mouse tissue, Juhasz *et al.* grafted human skin onto the flanks of athymic nude mice followed by injection of human melanoma cells into the graft [243]. Human melanoma cells formed more metastases from the graft than identically prepared cells injected into mouse skin. Another chimeric approach was used by Teraoka *et al.*[244] in a SCID mouse model of human lung cancer. They implanted human fetal and adult lung tissue into mammary fat pads and then analysed metastasis to both mouse and human lungs following subcutaneous inoculation of human squamous cell lung carcinoma and adenocarcinoma cell lines. Most (60–70%) mice had metastases in both mouse and human lung tissue. However, metastases formed only in fetal, not adult, lung tissue. This finding again emphasizes age-related factors when designing experiments of this type. Analogously, Schtivelman and Namikawa showed that small cell lung carcinoma cells appear to prefer human bone marrow as a site for bone colonization and human fetal lung in SCID mice to mouse bone marrow or lung, respectively [245]. These findings also make two important points: mouse skin is not the same as human skin, and tumor cells can tell the difference. Human tumor cells appear to have a predilection for human tissues when metastasizing. This has profound implications regarding experiments evaluating differential organ colonization of human cells in immunocompromised mice. Whether grafting human tissue into mice is practical for large-scale experiments remains undetermined.

The mechanisms by which orthotopically injected tumor cells metastasize more efficiently and colonize in a pattern reminiscent of human cancer remains largely a mystery. Clearly interaction with the host milieu is an important determinant of tumor cell behavior [246]. Miller and McInerney showed that mammary tumors grew preferentially upon implantation into the mammary glands of syngeneic mice compared to implantation subcutaneously [196]. This effect was lost if cells were injected into cleared mammary fat pads. Since both sites provide stroma, and the latter site lacks epithelium, an epithelial-derived factor was suspected. When epithelium was replaced, differential growth was again observed. Organ-specific responses to tissue factors have also been suggested. For example, human renal cell carcinomas produce less basic fibroblast growth factor when implanted into the skin compared to the same cells injected into the kidney [247,248]. The SW-1 variant of the murine K-1735 melanoma was isolated from an amelanotic lung metastasis and produces metastases in many organs. Interestingly, the tumors are all amelanotic, except those growing in the brain [249]. Both of these examples show clearly that tumor cells respond differently to the environment.

Although not directly related to formation of metastasis, the organ microenvironment can influence responses of tumors to chemotherapy as well. Slack and Bross showed that patient breast cancer metastases to regional lymph nodes and skin were more sensitive to drugs than lung or bone metastases [171]. Analogous results have been seen in experimental systems [250–254]. These results are not completely surprising, but they point out the need to consider sites of implantation and metastases if development of antimetastatic or antimetastasis therapies are the objective.

Clear association between propensity to metastasize and extent of neovascularization has been demonstrated in a number of human cancers and experimental models [19,25–31,255–258]. McCulloch *et al.* [92] propose that the shedding of tumor cells into the vasculature is proportional to the number of vessels within a tumor. This suggests that protocols using experimental models must accommodate current knowledge regarding regulation of angiogenesis (i.e. vessel source). Some considerations are described below.

O'Reilly *et al.* [159] pursued the finding that presence of large tumor mass could inhibit the growth of some clinical [259–262] and experimental

malignancies [263–267]. Their studies identified a soluble factor (angiostatin) in serum and urine which inhibited growth of metastases in some animal tumor models. Others presented evidence for secreted factors that stimulate [153,268] or inhibit [158, 269,270] tumor growth and/or invasiveness. Besides demonstrating cell–cell communication between tumor cells *in vivo*, these studies also identify several parameters that affect the design of *in vivo* studies.

Unless cell–cell communication is being tested, *in vivo* studies should never employ inocula into multiple sites of the same animal. In other words, there should be one tumor per animal. Growth rate [271,272], metastasis, drug sensitivity [273–277] and other phenotypes are modified by cells located at separate sites in the host. These variables are not always affected [278], however. Therefore, it is necessary to evaluate each cell line or tumor sample independently before attempting multiple injections or implantations into the same animal.

Also, introduction of multiple primary tumors effectively decreases the time allowable for metastases to develop. This is because total tumor burden will overwhelm the animal before macroscopic metastases can develop.

Spontaneous metastasis studies should incorporate two groups: animals in which the primary tumor remains throughout the experiment and animals in which the primary tumor is removed (to allow metastases to grow to detectable size). Of course, the timing for primary tumor removal is critical. In general, tumor size is proportional to the likelihood of developing metastases [154,231]. However, the threshold after which metastases are likely to be present must be determined empirically for every cell line and tumor. Unfortunately, practical considerations regarding the maximum size at which tumors can be removed without jeopardizing the health and recovery of the animal must be considered. We have found that a mean tumor diameter of approximately 1 cm is a good starting point for defining the time at which metastases take place. Larger tumors can be accommodated in rats.

Experiments measuring metastasis following removal of the primary tumor can be critical to claims about metastasis suppression. For example, we found that introduction of chromosome 6 into human melanoma cells [279] suppressed metastasis significantly. The nonmetastatic melanoma hybrids grew somewhat slower than the parental cell line, leaving the possibility that metastasis was not really inhibited. Rather, the growth was sufficiently slow that metastases could not be found in random microscopic sections. To obviate this interpretation, tumors were removed from nude mice at a time when most animals will already have occult, microscopic metastases (approx. 1 cm mean tumor diameter). The mice were then allowed sufficient time for metastases to grow. No metastases were ever seen. Similar studies have been done using other melanomas [280] and human breast carcinoma cells [281].

Injection into a relevant tissue is not sufficient to ensure reproducibility of metastatic potential. In rats and mice, the mammary fat pads are extensive and span nearly the entire length of the ventral surface. There are even vestiges of mammary tissue on the flanks and backs of rats and mice. Therefore, orthotopic introduction of breast tumor cell lines into animals could be done over a wide area. Data presented by Kyriazis and Kyriazis [189] indicate that there is a so-called 'cranial–caudal' gradient also playing a role in tumor behavior. Meschter *et al.* showed that mice bearing MDA-MB-435 tumors at the thoracic mammary fat pad developed more metastases than those with tumors at the inguinal mammary fat pad [136]. The pattern of metastases also changed. Tumors in the inguinal region produced extensive intra-abdominal lymph node metastases, whereas thoracic tumors developed more blood-borne metastases. Clearly, the frequency and location of metastasis is altered based upon implantation distance from the head [40]. Placement also will impact the ability to remove the locally growing tumor if needed.

Other factors influencing tumor growth and metastatic potential in experimental animals

One approach taken to improve the tumorigenicity and/or metastatic potential of primary explants of human cancers is to mix the cells with the reconstituted basement membrane mixture, Matrigel [282,283], or with normal fibroblasts [284,285]. Both approaches can increase 'take' rates by 2- to 10-fold. Co-injection of syngeneic embryo cells with B16 tumor cells resulted in more metastases than tumor cells alone [286]. Price [40] found that human breast carcinoma cells could increase both tumor incidence and growth rate in nude mice using Matrigel, fibroblasts or human breast epithelial cells. Tumors that resulted from the co-injections were more vascularized and had smaller areas of necrosis. Unfortunately, changes in metastatic potential were not reported. Some tumors and cell lines are unaffected by co-injection, however [279].

Mechanics of injections

Besides location of injection, the volume and the viscosity of the inoculum are important. For subcutaneous injections, volumes of 0.1–0.2 ml are sufficient with cell concentrations up to 1×10^8 cells/ml. For intravenous injections into mice or rats, a volume of no more than 0.2 ml is recommended. If larger volumes are injected, plasma blood volume exceeds the normal range such that the distribution pattern of cells is altered. This parameter is of particular importance if tumor cells are to be injected with other materials (e.g. drugs, peptides, etc.). Total volume of inocula must be calculated and should not exceed 0.2 ml for mature mice or rats. If the total volume exceeds this level, i.p. injection of non-cell inocula should be considered. Kinetics of distribution for i.p. injected materials are often superimposable to the kinetics following i.v. injection of liquids.

A restrainer in which the tail extends outside of the enclosure is required for i.v. injections. Several variations in design have been used successfully. The design I prefer is a hinged stainless-steel tube suspended on a pedestal. This unit is autoclavable; however, rinsing with a dilute bleach solution suffices for sterilization. A mouse is placed into the tube and enclosed while holding the tail. The dark environment has a calming effect. A lateral tail vein is identified and the needle is gently inserted to just below the skin. After 'tracking' for some distance to minimize leakage and backwash when the needle is withdrawn, the cells are injected using a 27-gauge needle fitted onto a 1 cc tuberculin syringe (some prefer a 26-gauge needle). As with all injections, the bevel faces upward. During the process, a slow, steady rate of injection is the objective. For i.v. injections, successful inoculation is evident by the lack of resistance during the process. If any resistance is felt, the process should be restarted. It is best to begin injections at the most distal part of the tail. If the injection is missed, one can proceed cranially. If one starts at the base of the tail, the effort cannot be redeemed without subsequent inocula leaking from the hole(s) generated by prior injections. Although i.v. inoculations can be done without the aid of procedures to dilate tail veins, the process is facilitated when tail veins are enlarged. This can be accomplished by dipping the tails into hot water, swabbing with irritants (such as xylenes) or brief warming under a heat lamp (preferred). We have found that a high-walled container is useful in order to decrease the chances for escaping mice. Use of a heat (infrared) lamp requires close monitoring to assure that the animals do not get overheated and that personnel are careful not to touch the bulb since second and third degree burns can result.

The methods for injection of cells into the vasculature are varied. Since mice and rats are the most commonly used experimental animals, injection into the lateral tail veins is the favored method. A common site for injection into hamsters is the penile vein. However, some laboratories expose other veins using simple blunt dissection techniques [287,288].

Injection into specific tissues requires empiric determination of injection volumes allowable. For the types of studies we have done, inoculum volumes of 100 μl for mammary fat pad injections, 50–100 μl for intrasplenic injections, 25–50 μl for intraadrenal injections and 50–100 μl for intradermal injections work best. If the volume is too large, there is significant leakage into surrounding tissues as well as significant damage to normal stroma and epithelium. If the volume is too small, accuracy of injection becomes difficult to control. For volumes less than 100 μl, accuracy of delivery volume is best accomplished with a sterile Hamilton syringe or tuberculin syringes.

Injections into the subcutaneous or intradermal sites do not require the animals to be anesthetized; however, intradermal injections are much easier when the animals are unconscious. Comparison of metastasis assays using identically prepared cells in anesthetized animals vs unanesthetized animals yielded identical results with the 13762NF rat mammary adenocarcinomas, C8161 melanoma and B16 melanoma. We have used methoxyflurane (Metofane, Pitman-Moore) inhalation anesthetic successfully. Simply place a small volume of Metofane under a wire screen suspended above the floor of a container with a lid. Rats and mice will become unconscious within a couple of minutes and the effects will last less than 5 min. Be sure to construct the container so that none of the animal touches the liquid and avoid personnel exposure. It is a good idea to utilize mesh that allows feces to drop as this maintains clean conditions.

For injection into other sites (mammary fat pad, intrasplenic, other orthotopic sites), a mixture of Ketaset–Rompun (Ketamine–HCl, xylazine) injected intramuscularly provides excellent results. This anesthetic is also useful for simple surgical procedures like tumor removal. A stock solution of 10 ml of Ketamine (100 mg/ml) containing 1.6 ml of xylazine (20 mg/ml) works well for rats. A female Fisher 344 rat weighing 150–180 g inoculated i.m. with 0.1–0.15 ml will remain unconscious for 1–4 h. For mice, the stock solution should be diluted 1:10 in saline. For

most mice, we have found that 0.1 ml/10 g body weight is sufficient to anesthetize for 30 min to 1 h. However, nude mice require a higher dose (0.15 ml/10 g body weight). The reasons for this difference in dosage are not completely understood.

Finally, most investigators utilize cells grown in tissue culture for injection; however, some tumors have not adapted well to *in vitro* growth. Therefore, it is necessary to serially transplant tumor tissue. Several methods have been used to accomplish this. Basically, the tumor is removed before it has developed extensive necrosis and viable tumor tissue is dissected away from normal or dead tissue. Tumor tissue is prepared for transplant by mechanical or enzymatic dissociation before placement into another host. Some preparation techniques will be described below.

Following injection or implantation, mice should be monitored daily. The length of an experiment depends upon multiple factors; however, animal welfare is of paramount importance. On or before the first signs of distress (irregular behavior, lethargy, circling, weight loss, masses), the animals should be euthanized.

Enumeration of metastases

For some studies, presence or absence of metastases is sufficient; however, quantification of metastases is often desired. To assess metastasis, animals are killed, organs are examined then removed and rinsed in cold water to remove excess blood.

The method of euthanasia affects the ease by which metastases can be detected and quantified. We have used three approved methods of euthanasia: carbon dioxide, cervical dislocation and overdose using anesthetics. All methods for euthanizing animals must be approved beforehand by Institutional Animal Care and Use Committees (IACUC). Guidelines for euthanasia are evolving; so, regular consultation with veterinarians concerning procedures is advised.

Although efficient, fast and inexpensive, the use of carbon dioxide is not recommended because of a substantial number of petechia in the lungs. While tumors are often colored differently, presence of hematomas complicates counting, particularly for less experienced lab personnel. Cervical dislocation works well but sometimes can result in the presence of clots in the lungs. Nonetheless, we use cervical dislocation as the primary method for mouse euthanasia (see below). For rats and as an alternative for mice, Metofane inhalation works extremely well. The animals fall asleep and then die. Lungs are clear and quantification of metastasis is unimpeded. The problem is expense.

Identification of macroscopic metastases is easier if coloration is different from parenchyma. This was a major advantage when studying melanoma; however, this characteristic was not available for other tumor types. It is possible to identify metastases in most tissues. They appear as clear or white raised gelatinous surface structures. Examination is facilitated by the use of a dissecting or stereomicroscope. However, if there are imperfections in the tissue or if the tumor is small, errors can occur. Therefore, different approaches have been employed to enhance visual contrast between tumor and parenchyma.

For lung metastases, Wexler exposed mouse trachea and injected 2 ml of a 15% solution of India ink in neutral buffered formalin. The trachea was sealed with surgical suture to prevent leakage; the lungs were dissected and placed into a beaker of tap water. Tumor colonies were bleached with Fekete's solution. Metastases appear as white colonies against a black background. While this approach works well, it can be impractical when lots of animals are being necropsied simultaneously. Also, it can be done more easily with assistance. Therefore, the preferred method is fixation of organs and tissues in Bouin's solution. After fixation, the tumors appear as white or pale yellow spots against a darker yellow background. The use of Bouin's is not without problems, however. Tissues become brittle making subsequent confirmatory histology difficult. To partially alleviate this problem, we use a mixture of one part Bouin's fixative in five parts neutral-buffered formalin.

One important consideration when removing tissues is to avoid touching the surface with forceps. The striations caused by the teeth can complicate visualization of small metastases. Most lung [165, 289–291] and liver [292] metastases develop near the surface; therefore, anything which compromises visualization will affect quantification ability. Similar precautions should be taken during the preparation of other tissues.

Random samples of tissues should be submitted for histologic confirmation of presence/absence of metastases. Ideally, one could quantify metastasis by serially sectioning tissues, measuring the surface area of the lesions, calculating total tumor volume and comparing that to the organ/tissue volume [293]. Unfortunately, this is impractical. The use of histologic preparations allows determination of total tumor burden. Most investigators merely count the number of metastases. Small lesions are not equivalent to large lesions, however. Assessment of

metastatic tumor burden should not only include assessment of number, but also size/volume of lesions. In the lung, the majority of metastases are spherical [165] making this calculation straightforward. Side-by-side comparison of the number and volume of metastases can give important information regarding the mechanism responsible for developing metastasis by different cell lines.

Another method for estimating the number of metastatic cells successfully colonizing a tissue is accomplished using tumor cells tagged with a drug resistance, genetic or color marker. Visualization of metastases can be enhanced if the cells are tagged with the lacZ gene [53,294–298] or fluorescein [173]. The tumor cells appear blue or yellow-green, respectively. However, stability of the transfectants *in vivo* can vary considerably and a substantial proportion of macroscopic metastases will no longer be colored or the metastases will contain mixtures of colored and colorless cells. Therefore, if coloration were used as a criterion, the number and volume of metastasis would be underestimated [298]. If tumor cells are tagged with a drug resistance or genetic marker, cells colonizing different organs can be recovered from dissociated tissues [299] and the proportion compared to the inoculum. Similarly, cells labeled with ^{125}IUdR or BrdU can be detected in dissociated tissues using a gamma counter or ELISA, respectively [298]. To use these approaches, prior verification that the label does not affect biological behavior of cells must be obtained.

Counting and measuring metastasis is a well-established and reliable technique, but it can be tedious. More importantly, it is not well-suited for large-scale experiments, such as drug screening for antimetastatic compounds. In an effort to streamline the experiments, Paul Aeed and I evaluated the utility of using organ weights as a method for quantifying metastasis in three metastasis models (13762NF rat mammary adenocarcinoma, B16 melanoma and RAW-117 lymphosarcoma). Organ indices (organ weight/total body weight) were determined following removal of excess tissue prior to placement in Bouin's fixative for counting/measuring metastases as above. Correlations between organ index (i.e. normalized to animal body weight) and number of metastases or metastatic volume were calculated using Pearson's correlation coefficient (r).

We first examined the criterion of lung weight (Note: not index) as a measure of B16-F10 experimental metastasis and obtained poor correlation ($r = 0.23$), due to high variability of lung weights. To reduce variability, lung index was calculated. The correlation improved ($r = 0.50$). The correlation between lung index and number/volume of metastases formed by 13762NF cell clone MTLn3 was 0.71–0.75, respectively (Figure 1). However, when the number of metastases per lung was ≤ 100, the correlation dropped to 0.37. The correlation dropped further ($r = 0.20$) when the number of metastases was ≤ 50. These r values implied that lung index becomes less sensitive as metastatic tumor burden decreases. Similar trends were observed in the B16-F10 system (Figure 2). Since most experiments yield < 100 metastases, weighing lungs is of insufficient sensitivity for detection.

Liver weight indices correlated well with other methods of metastasis quantification using the RAW-117 lymphosarcoma ($r = 0.80$) (Figure 3). As with the lung indices, however, results were significantly affected by treatment with different drugs or treatment regimens. Overall, the organ index failed as a reliable measure of metastatic tumor burden in these three metastasis models. The failure was either due to lack of sensitivity (lung) or the fact that other treatments could alter the index (lung and liver). It should be noted, however, that organ weight and metastatic tumor burden correlate well in other models (e.g. the Lewis lung carcinoma [300]). Therefore, use of organ weights as a measure of metastasis must be confirmed on a case-by-case basis.

Table 1. Correlation between the number of metastases and organ weight index

Cell line	No. of metastases (range)	Organ	Pearson's correlation coefficient (r)
13762NF clone MTLn3	0 to >500	lung	0.71
13762NF clone MTLn3	0 to ≤100	lung	0.37
13762NF clone MTLn3	0 to ≤50	lung	0.20
B16 melanoma subline F10	1 to >250	lung	0.50
B16 melanoma subline F10	0 to ≤50	lung	0.22
RAW117 lymphosarcoma subline H10	1 to >500	liver	0.80

Organ index = weight of an organ/total animal weight.

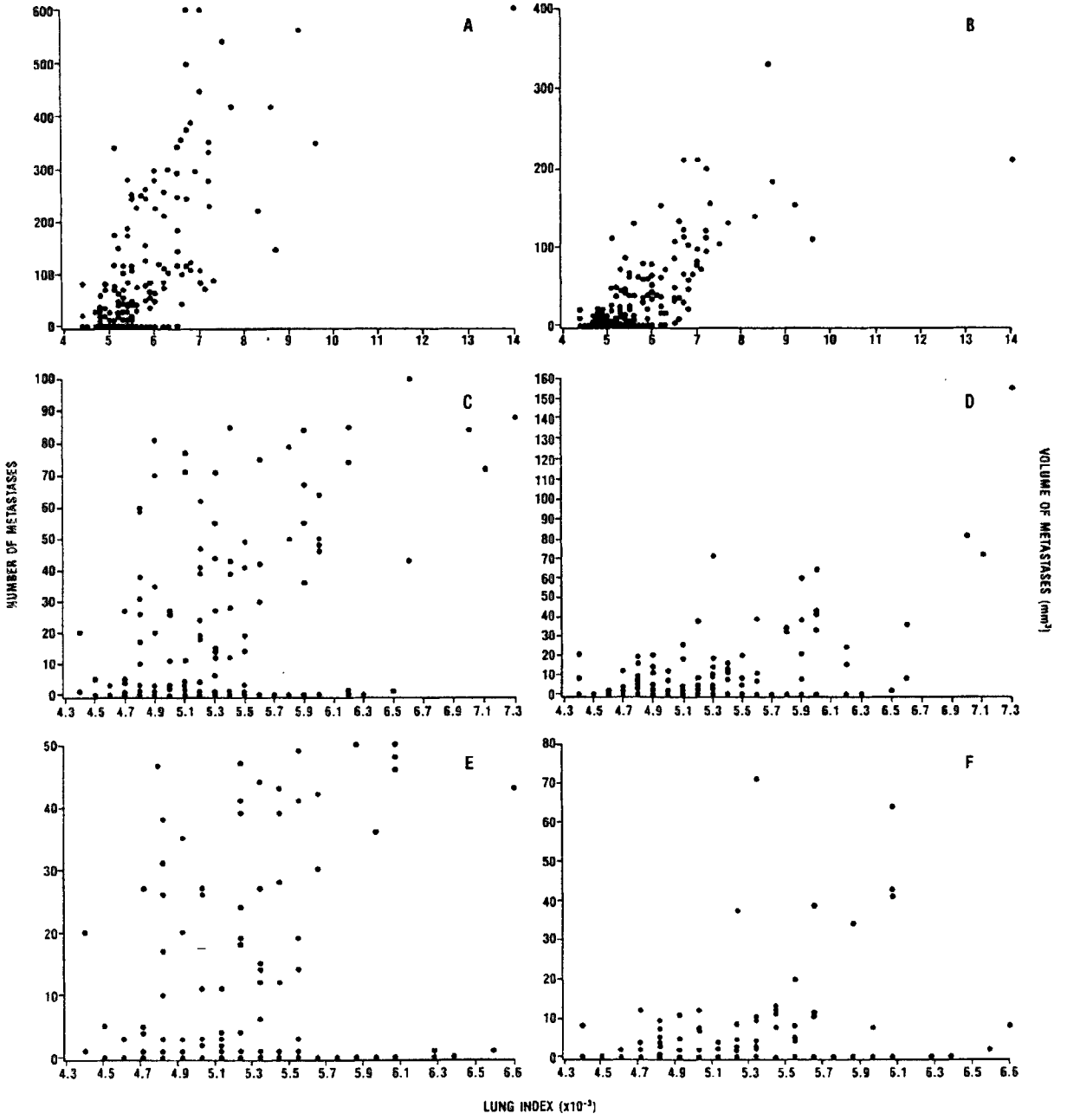

Figure 1. Comparison of the number (left panels) or volume (right panels) of lung metastases formed by 13762NF rat mammary adenocarcinoma cell clone MTLn3 with lung index (lung weight/total animal weight). (A,B) All rats. (C,D) Rats with ≤ 100 lung metastases. (E,F) Rats with ≤ 50 lung metastases. Pearson's correlation coefficients are found in Table 1. These results indicate that weighing lungs is of insufficient sensitivity to quantify metastases in this model.

Measurement of tumor growth

Local tumor growth rate during metastasis experiments should be monitored carefully. However, measurement of *in vivo* growth is 'fraught with low power (inability to detect differences because of poor experimental design) or misleading type I error rates (false positive result)' [278].

We and others have struggled to identify the best method for determining tumor volume. Difficulties arise because tumors have irregular three-dimensional shapes. These issues were addressed

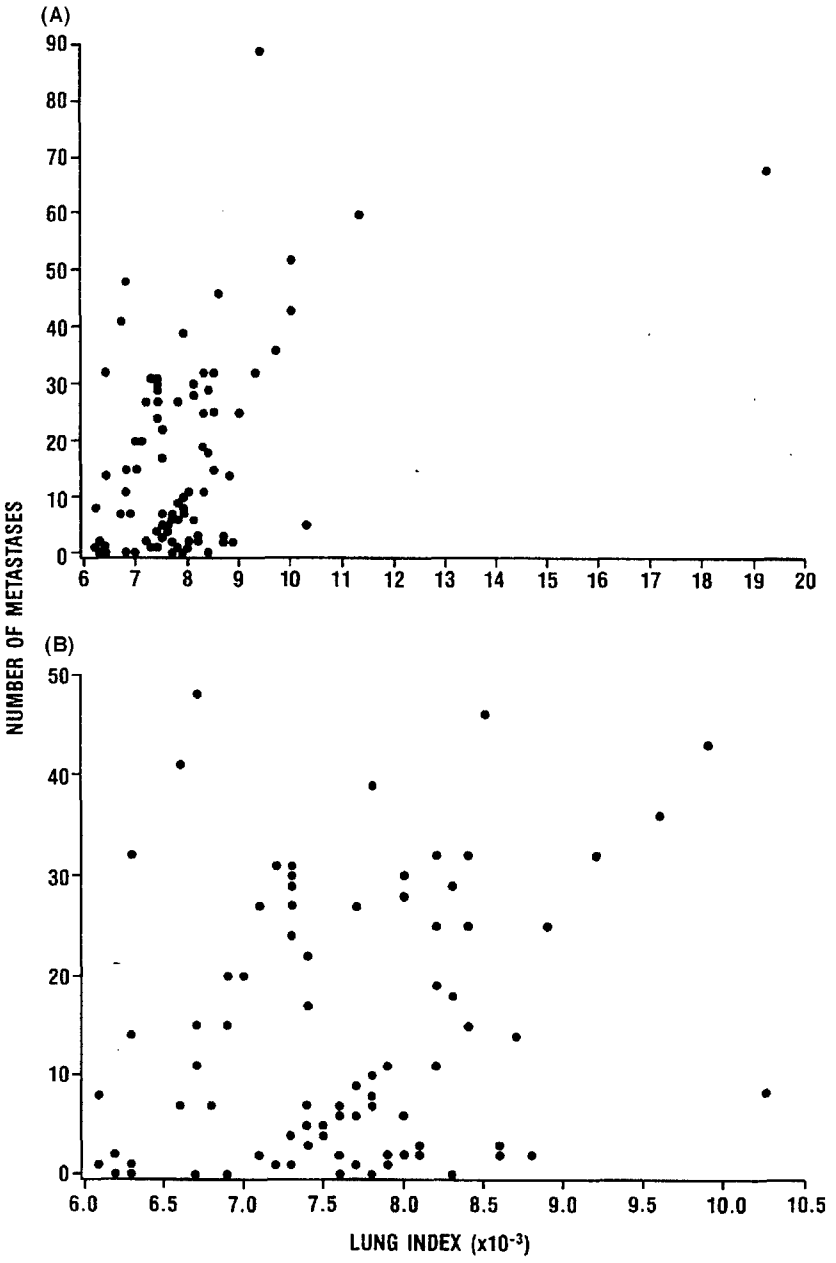

Figure 2. Comparison of the number of lung metastases formed by B16-F10 murine melanoma cells with lung index (lung weight/total animal weight). (A) compares metastases in all mice and (B) compares only mice that have ≤ 50 lung metastases. Pearson's correlation coefficients are found in Table 1. These results indicate that weighing lungs is of insufficient sensitivity to quantify metastases in this model.

elegantly by Pimm and Morris [301]. The standard for determining tumor volume is liquid displacement; however, this requires removal of the tumor from the animal as well as dissection from other tissues. Therefore, interim measurements in a time-course experiment are not possible. They found that 'tumor volumes bore a better relationship to weight,' but weighing tumor also requires removal from the animal. One laboratory of which I am aware even tried to estimate tumor size by weighing the experimental *animal.* The latter approach is seriously flawed because body weight also changes during the course of an experiment, particularly if the tumor causes cachexia. Likewise, there is no method to normalize the results to normal weight gain/loss during the course of an experiment. Since determining volume and weight are impractical for many studies, calculation of tumor volume based upon shape represents a better strategy.

Using a variety of formulae, Pimm and Morris concluded that multiple different methods could be employed to estimate volume. However, all over- or under-estimated the true volume. Despite these types of errors, each could be useful for comparing

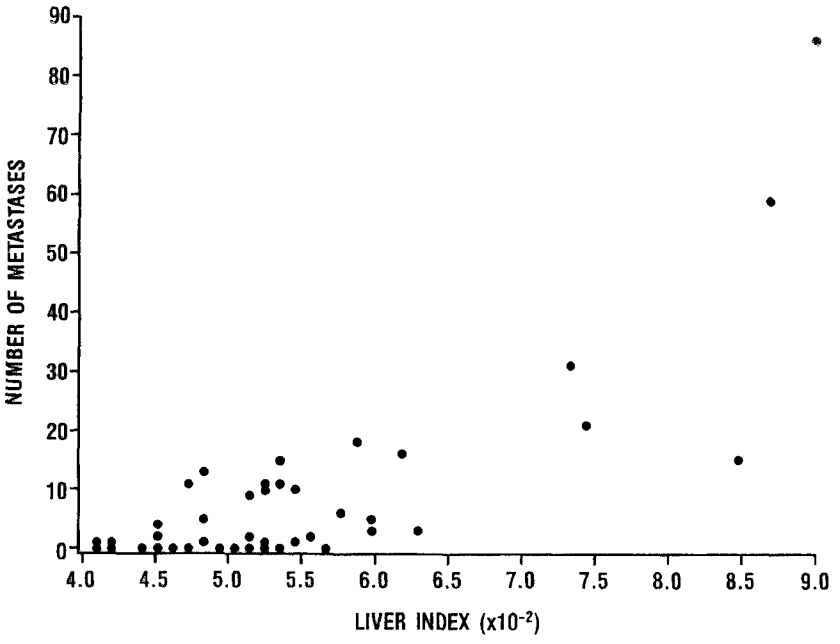

Figure 3. Comparison of the number of liver metastases formed by RAW-117-H10 murine lymphosarcoma cells with lung index (lung weight/total animal weight). Pearson's correlation coefficients are found in Table 1. These results indicate that weighing liver is a reasonable method to quantify metastasis in healthy mice.

growth rates of different tumors as long as a single method was used throughout an experiment [301].

We estimate tumor volume using a formula for mean tumor diameter (MTD). MTD is the square root of the product of orthogonal measurements. We do not try to measure the tumor in three dimensions because different personnel cannot obtain the same result with the depth dimension. Moreover, unless the tumor is 'fixed' into a position, the additional manipulation required to measure the tumor depth should be avoided.

Heitjan *et al.* [278] recently compared statistical methods for analysing tumor growth of different cell lines and/or experimental conditions. Rather than reiterating their conclusions and recommendations, it is important to highlight a common mistake made by investigators, that of comparing tumor diameters at particular times during a continuum (e.g. MTD on day 46 post-inoculation). This practice ignores basic assumptions of the statistical models, thus making such comparisons invalid.

Statistical considerations

One thing that new investigators fail to recognize when doing *in vivo* experiments is the inherent variability, even in genetically identical animals. As a result, subtle differences in experimental outcome are often indistinguishable from background 'noise'. Therefore, experimental design must accommodate the statistical considerations. For most metastasis studies, groups consisting of a minimum of 8–10 animals are required. Sometimes, even more animals are required. The total number of test animals needed can be determined using appropriate power calculations [278].

Preparation of cells for metastasis assays

As stated above, the most critical factor when performing metastasis experiments is availability of metastatic cells. The source of cells varies widely. Most laboratories use established cell lines that are maintained in tissue culture. However, some experiments can be done with freshly prepared transplant tissue from tumors grown *in vivo*. Most of the discussion below focuses on techniques used for properly preparing cells from tissue cultures. However, it is to be emphasized that use of any tumor cell should be preceded by thorough analysis and characterization of the cells with regards to the experimental parameters under which the experiments will be performed. Species of origin should be verified by karyotype and/or isoenzyme analysis. Tissue of origin should be verified by at least one tissue-specific marker.

Preparation of cells from tumor tissue

It is essential that only sterile, viable, and non-necrotic (which appears shiny, often pearly white, white or clear gelatin) tumor tissue or cells be used. To isolate tumor cells from tumor masses, peripheral tissue is often the best source since tumors

usually grow more actively around their periphery [302,303]. Once viable tissue is dissected away from the mass, it can be dissociated using scissors or scalpels, forcing through a stainless-steel sieve using a syringe plunger, placement in a Stomacher® device, or digested using collagenase, trypsin or other proteinases (e.g. dispase) (reviewed in [299]). Mechanical and enzymatic digestion can be combined. For example, some labs have successfully used dissociated B16a tumors in combination followed by centrifugal elutriation to prepare cells for injection and transplantation [304,305]. Viability is then determined by Trypan blue dye-exclusion and cell number determined by hemacytometer or Coulter counter. Reviews regarding preparation of tumor cells from *in vivo*-derived tissue are available [306–312].

We have found that the following technique works well for most applications. Using this approach, we have successfully isolated tumor tissue for transplantation and establishment of new cell cultures. Tumors are removed and immediately placed into sterile 60 mm tissue culture dishes. Under laminar flow, the tissue is dissected and viable, nonnecrotic tissue is isolated. Using sterile forceps, individual pieces of tumor are rinsed in excess ice-cold saline solution 10 times. This is followed by similar washing in serum-free tissue culture medium containing 2 × your favorite antibiotic (we generally use gentamycin sulfate). The tumor pieces are placed into approximately 0.5 ml of serum-free medium and minced using sterile scalpel blades until they are sufficiently small to easily pass through the bore of a standard tissue culture pipet. The mixture is then aliquoted into 60-mm dishes containing whatever tissue culture medium is desired without antibiotics. The dishes are left untouched for 2–3 days before the medium is aspirated and replaced (for presumed adherent lines). This procedure allows the cells to acclimate and begin migrating onto the tissue culture plastic. The aspiration should be sufficiently gentle to avoid loss of weakly adherent cells.

Use of antibiotics is not continued for any cell cultures in my lab. Many antibiotics alter cell surface properties of eukaryotic cells. Since metastatic potential is determined in large part by cell surface characteristics [313,314], we have chosen not to alter conditions further by inclusion of antibiotics. Furthermore, even low level bacterial contamination can result in production of immunomodulatory molecules (e.g. lipopolysaccharide in the tissue culture medium) which can, in turn, affect metastatic properties. I frequently find that cultures received from laboratories routinely using antibiotic-containing media often have low-level (often drug-resistant) contamination that becomes apparent upon removal of the antibiotic.

Whenever using fresh tumor tissue, it is especially important to recognize that tumor cell suspensions or minces are notoriously heterogeneous. One needs to plan accordingly. Failure to do so will result in even greater artificial selection than already occurs during manipulations. The reasons for this are several-fold. First, tumors are not homogeneous. Even cursory examination of tumor sections reveals zones of different morphology [315]. Transplantation or isolation of one zone without the others represents an artificial selection. Second, not all tumors can be uniformly dispersed due to stromal–parenchymal interactions. Correspondingly, the ratio of stroma to parenchyma may be altered during dissociation. Third, the ratio of viable to nonviable cells varies between preparations and techniques. Fourth, even when single cell suspensions have been prepared, they are not always uniform. And fifth, there is almost always host cell contamination. Therefore, preparations from fresh tumor mince should not be utilized for quantitative studies. However, they are perfectly acceptable for qualitative metastasis studies.

Cell or tissue culture considerations

The use of *in vitro* cultured cells avoids many of these limitations. Cells can almost always be evenly dispersed. The viability should be uniformly high. Suspensions are generally more uniform, so 'homogeneity' is more likely. Use of cultured cells allows standardization of animals for experiments (age, sex, strain, injection on the same day and time, etc.). Finally, host cell contamination is avoided. Of course, tissue culture imposes its own set of selective pressures and caveats impacting experimental design.

A cardinal rule for growing cells in culture is: do not contaminate the culture. As with use of tumor tissues, cell cultures must be sterile. There should be no contamination, either grossly or microscopically. Cultures should be checked regularly and found free of *Mycoplasma* contamination. Presence of microorganisms in cell preparations can significantly influence metastatic potential. Cross-contamination with other cells must be avoided for similar reasons. To eliminate the possibility of mixing cells, only one cell line or clone should be taken into a laminar flow hood at any time. And materials or reagents used during cell culture for one cell line should not be used for other cultures.

The health of cells is of paramount importance. Other activities should be arranged around the experiment, not vice versa. To illustrate, I once visited a lab in order to teach some of the techniques involved in metastasis assays. The fellow preparing cells for injection committed a major tissue culture sin. I was surprised to find that 15 min after our group returned from lunch, the cells were ready for injection. Knowing that the harvesting procedure takes longer than this, I questioned the fellow. Apparently, the cells had been detached, counted and inocula prepared prior to lunch. The vial containing the suspension was then placed into a refrigerator for the hour. Taking advantage of a 'teachable moment', we examined the suspension. The viability had dropped to approximately 50%, so, the experiment was postponed. This senior fellow had apparently been doing cell culture in this manner throughout training unbeknownst to the advisor. Inconsistent results of the past could then be explained.

Phenotypic instability and drift

Tumor populations display greater genetic instability than their normal counterparts. As a result, it is common to observe phenotypic drift during both *in vivo* and *in vitro* passage (reviewed in [9]). There is often a gradual change over time, presumably as proportions of different clones change [316,317]. As with all tumor phenotypes, propensity for clonal drift is heterogeneous. Some cells drift readily, whereas others are more stable. The reasons are unclear. However, intrinsic genomic instability is coupled with selection pressures encountered during growth. It is a myth that drift occurs at faster rates in cell culture than in animals. For the most part, phenotypic drift is cell-dependent. When precautions to minimize selection are not followed, artificial (i.e. iatrogenic) drift can be expected.

To minimize the impact of phenotypic drift, a baseline behavior for each cell line at a given passage must be established followed by periodic assessment of biological properties at regular intervals. If behavior changes (i.e. metastatic potential increases or decreases, or if distribution of metastasis is altered), frozen aliquots from a lower passage should be retrieved and used. As long as metastatic potential does not change within a given interval of growth, use of cells for *in vivo* assays is acceptable. The difficulty with this approach is distinguishing typical variability of *in vivo* studies from clonal drift.

The simplest method for keeping track of cell divisions is to monitor passage number. As long as conditions used for passaging cells are consistent, a reasonably accurate accounting of cell divisions can be calculated based upon doubling time, saturation density and split ratio. Minimizing phenotypic drift during *in vivo* growth is accomplished by using the techniques described above. Analogous procedures should be incorporated during cell culture.

Homogeneous mixing

Even in cultured cells, absolute homogeneity of a mixture is virtually impossible. That tumor cells are more genetically unstable than their normal counterparts amplifies the impact of phenotypic drift seen in normal cells [9]. So, by the time there are sufficient cells for doing an experiment, it is likely that clonal populations are no longer homogeneous. As a result, thorough triteration of cell suspensions when subculturing or preparing cells for injection is required in order to ensure even distribution of cells. This presupposes that artificial selection of easily detached cells has not already taken place.

Cell viability

Never use cells for *in vivo* studies that are less than 90% viable. Dead cells do not grow to form macroscopic metastases; however, they can modify the behavior of viable cells. Using B16 melanoma cells, Fidler injected 50,000 viable cells intravenously, resulting in approximately 40 metastases per mouse. Injection of 100 000 viable cells produced just over 200 metastases. Mice co-injected with 50 000 live cells and 50 000 lethally irradiated cells developed 160 metastases, whereas 50 000 irradiated cells injected alone formed no metastases [312]. Clearly, the presence of dead cells influenced the metastatic efficiency of the live cells. Although not part of this published experiment, smaller proportions of dead cells could also cause changes in metastatic potential.

Notice also that there is not always a linear correlation between cell number injected and the number of metastases formed. For example, doubling the number of tumor cells injected does not always translate to twice the metastases. In some cases it may be more, in some cases it may be less.

Use of single cell suspensions

Incidence of metastasis is proportional to the number of viable tumor cells entering the circulation [290,312], but mere presence of tumor cells in the blood does not always portend development of

metastases [170]. The number of metastases formed also varies by the number of tumor cells present as emboli in the blood of patients [307,308,311]. Fidler [312], Liotta *et al.* [318] and Updyke and Nicolson [319] showed that presence of multicellular emboli increased the frequency of metastatic colonization in experimental systems. Updyke selected for variants of the B16 melanoma with propensity for homotypic aggregation. The variants had concomitantly higher efficiency of metastatic colonization. Thus, the method of preparation is important to ensure that single cell suspensions are prepared. What happens once the cells are injected cannot be controlled.

The proportion of single cells versus multicell clumps is influenced by the methods used in preparing cells for injection. Most clumps can be dissociated by gentle pipetting. The proportion of single cells is enhanced when ice-cold media or saline is used. Also, the use of a smaller bore pipet is useful, but the use of these must be exercised with caution since too small a diameter can result in cell killing. Cell aggregation is a time-dependent phenomenon; therefore, it is imperative that syringes containing cell suspensions be prepared immediately before injection, not prior to the animals being ready. Likewise, it is important that a filled syringe not be allowed to sit for extended periods prior to injection, even if placed on ice.

When loading a syringe, it is important that no needle be in place. Drawing a cell suspension through a needle with negative pressure results in significant killing. These types of results are cell line-dependent. More or less extensive killing will occur for other cell lines and other conditions. The point is that this parameter must be considered important until proven otherwise.

Once fluid is drawn into the syringe, the needle should be placed onto the syringe and aligned with the gradations. Alignment makes the process of injection easier since one can readily see the amount injected without having to contort. Analogously, passage through a too small needle bore causes cell injury and death. We routinely use 27-gauge needles without major problems; however, 30-gauge should be used with greater caution. The rate of injection also influences trauma using different size needles. Slow, steady injection overcomes many of the problems associated with a smaller bore.

Methods for harvesting cultured cells

There are several methods for passaging cells and for obtaining cells to be used for animal injections. For suspension cultures, dilution of single cell suspensions results in effective subculturing. For adherent cultures, other methods must be employed. Scraping cells followed by gentle pipetting, filtration and/or sedimentation has been used to obtain cell suspensions [107]. However, yields using this approach are inconsistent, and inter-experimental variability is high in my experience. Moreover, viability is usually lower than when enzymatic or chelating approaches are used. I believe this is due to the mechanical forces being applied. I have also had difficulty obtaining predominantly single cell suspensions using this approach.

Enzymatic or chemical detachment is more commonly used. Trypsinization or EDTA treatment can be used to rapidly and reproducibly detach cells from a substrate. The time required for detachment varies considerably from less than 1 min to more than 1 h, depending upon the cell line and culture conditions. Unnecessarily prolonged treatment of tumor cells affects survival and metastatic potential [320]. Conversely, failure to remove all cells from a plate imposes a selection for weakly adherent cells which have different survival, growth and metastatic potentials than strongly adherent cells [321–327]. Routine viability tests (Trypan Blue dye exclusion or MTT assay) or even plating *in vitro* (colony formation assays) can be used to control, insomuch as possible, the conditions prior to injection. Unfortunately, these tests do not always predict outcome *in vivo*.

The method of detachment profoundly influences metastatic potential of some cell lines. To illustrate, I will describe results using 13762NF rat mammary adenocarcinoma cell clones (Table 2). Highly metastatic cell clone MTLn3 produces more than 200 lung colonies following intravenous injection if trypsin (0.25% in calcium- and magnesium-free Dulbecco's phosphate buffered saline) is used to detach the cells. If EDTA (2 mM in calcium- and magnesium-free Dulbecco's phosphate buffered saline) is used, the average number of metastases formed drops to 50. A mixture of trypsin (0.125%) and EDTA (2 mM) results in an intermediate number of metastases. The viability was the same for all groups. Use of EDTA itself does not necessarily obliterate metastatic potential in all cell lines. Cell clone MTF7 produces more metastases when EDTA is used than when trypsin is used. Trypsin–EDTA solution gives results nearly identical to the use of trypsin alone (Table 2). These data demonstrate the absolute requirement to use recommended procedures for cell detachment. Otherwise, metastatic properties can be significantly altered.

Table 2. Effect of different detachment procedures and temperature on experimental metastatic potential[a] of 13762NF mammary adenocarcinoma cell sublines and clones

Cell line	Detachment procedure[b]	Temperature (°C)	No. of lung metastases[c]	Volume of lung metastases (mm^3)[c]
MTLn3	Trypsin	4	216 ± 32	155 ± 27
		25	282 ± 74	280 ± 56
	EDTA	4	50 ± 17	34 ± 13
		25	128 ± 35	107 ± 62
	Trypsin–EDTA	4	124 ± 25	99 ± 22
		25	NT[d]	NT
MTF7	Trypsin	4	111 ± 13	28 ± 4
	EDTA	4	343 ± 40	126 ± 31
	Trypsin–EDTA	4	94 ± 15	21 ± 4

[a] Cells were detached from tissue culture plates as described below and injected into the lateral tail vein of syngeneic, age-matched F344/NHSd female rats. For temperature determinations, cells were allowed to equilibrate for 10 min prior to beginning injections. Twenty-three days post-inoculation, rats were sacrificed and the number and volume of surface lung metastases determined by the method of Welch *et al.* [165].

[b] Detachment using trypsin = 0.25% trypsin in CMF-DPBS, EDTA = 2 mM EDTA in CMF-DPBS or trypsin–EDTA = 0.125% trypsin and 2 mM EDTA in CMF-DPBS.

[c] Values are mean ± SEM.

[d] NT = not tested.

Following detachment, cells should be maintained under minimal contact with polystyrene or tissue culture plastics. The reason is that adhesion to these surfaces occurs within 30 min. If cells are allowed to sit, some losses will occur, mostly for highly adherent subpopulations. The result is a solution from which fewer cells than expected will be injected. We have found that keeping cells in polypropylene tubes on ice minimizes cell loss while maintaining high viability. The use of ice is critical since allowing cells to return to room temperature increases the proportion of multicell emboli with a corresponding increase in number of experimental metastases formed (Table 3).

Culture confluence affects metastatic potential

As described above, the capacity of tumor cell lines to metastasize is determined, in part, by traits that are stable over many cell generations, and partially by traits that are acquired by adaptation or phenotypic instability [9,48,165,328–331]. We also reasoned that metastatic potential may be due to traits that change over less than one cell generation. This concept was supported by findings in the 13762NF rat mammary adenocarcinoma and B16 and K1735 murine melanomas [332]. Briefly, the ability to colonize lungs following intravenous inoculation increased as cells became more confluent. Confluence was defined as the maximum number of cells able to fit into a defined area prior to changes in cell cycle distribution. While changing confluence did not qualitatively alter metastatic potential (cancer cells must still possess all of the machinery necessary to complete all steps of the metastatic cascade), modification of metastasis in a quantitative manner suggested that some properties pertinent to metastasis are transient and manipulatable.

Such results show that the condition of cells prior to detachment must also be carefully controlled. Injection of MTLn3 cells (5×10^4) produced an average of 89, 141, 245 and 553 metastases at 20, 50, 70 and 100% confluence, respectively. B16-F10 cells (1×10^5) injected intravenously developed a median number of 15, 43 and 57 metastases at 20, 50, 70% confluence, respectively. Injection of K1735-M2 cells (1×10^5) yielded medians of 82.5, 169.5 > 200 and $\gg$ 200 surface lung lesions at 20, 50, 70 and 100% confluence, respectively. Changes in lung colonization were not due to simple passage or subculture since 'overgrown' cultures (e.g. 70% rather than 50% confluent) could be reseeded and grown to 50% confluence and the cells behaved as unpassaged 50% confluent cultures (Figure 4). Our experiments also demonstrated that the confluence-mediated effects on metastasis were not solely due to cell contact since cell-free conditioned media transfer could mimic these results [332] (Figure 5).

Roles for cell density and confluence have been implicated in various stages of tumor progression previously. Grundel and Rubin [333] have found

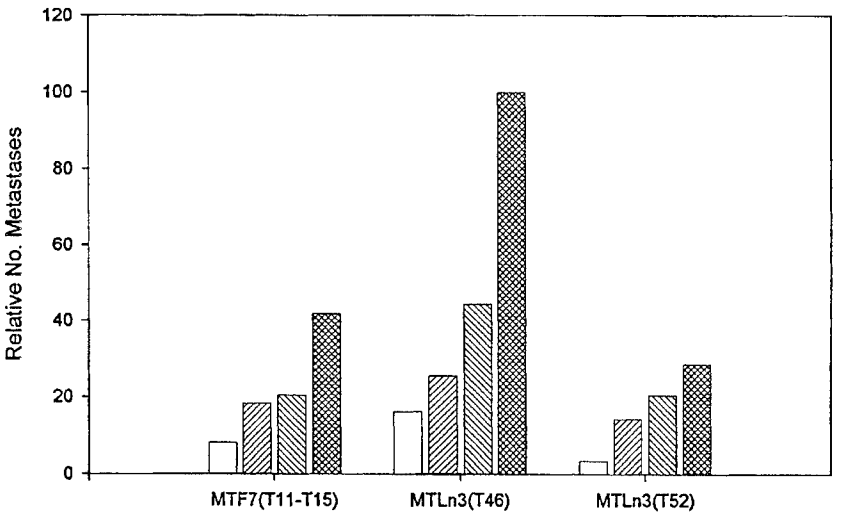

Figure 4. Relative metastatic potential of 13762NF rat mammary adenocarcinoma cell clones grown to different levels of confluence prior to detachment for experimental metastasis assays. Some data are from Ref. [332]. □, 20%; ▨, 50%; ▧, 70%; ▩100%.

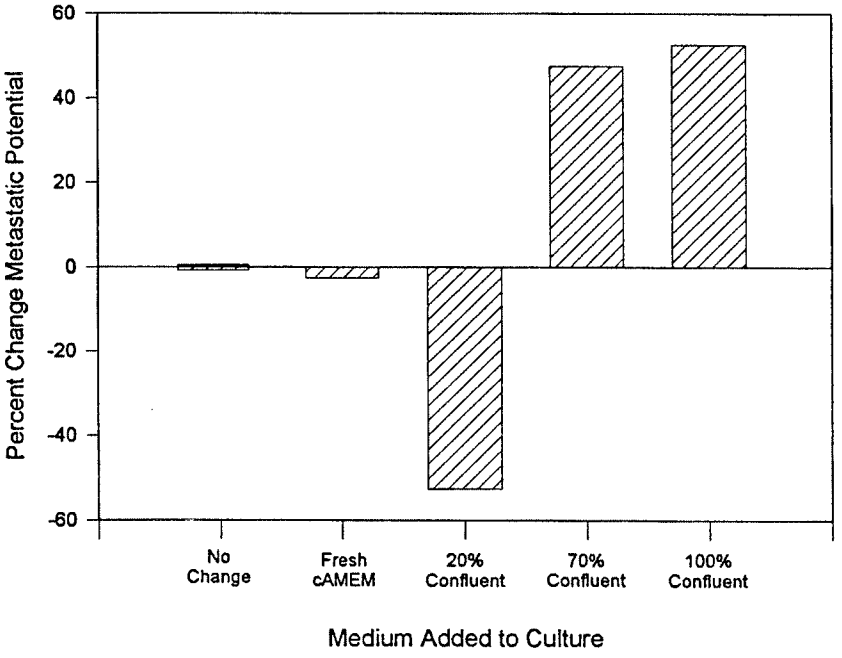

Figure 5. Percentage change of experimental metastatic potential of 13762NF rat mammary adenocarcinoma cell clone MTLn3 following exposure to conditioned media collected from MTLn3 cells at various levels of confluence. Conditioned medium was collected following 48 h continuous culture from cells seeded to yield levels of confluence shown at the time of media collection. Recipient cultures were seeded 48 h previously to yield 50% confluent cultures. Conditioned medium was added for 4 h prior to preparation for experimental metastasis assays. Note: if medium was not replaced or if fresh alpha-modified MEM supplemented with 10% fetal bovine serum (cAMEM) was added, metastatic potential was unaffected. Conditioned medium from a 20% confluent culture appears to suppress metastasis of 50% confluent cultures, whereas conditioned medium from 70% and 100% confluent plates increases metastatic efficiency. These data suggest the presence of paracrine metastasis-regulatory signals in the 13762NF model.

Table 3. Metastasis assays: recommendations and technical considerations

Animal health issues	Animal type issues	Injection issues	Cell health issues	Cell preparation issues	Experimental design

Adhere to AAALAC and AALAS recommendations for care and maintenance of research animals.	Use animals with MHC haplotypes as close to cell haplotype as possible.	Injection site should be identical between animals:
		• tissue of injection (orthotopic vs ectopic)
		• location along cranial--caudal gradient
		• volume of injection
		  • avoid tissue damage
		  • avoid changing blood volume
			Contact with animals should be done after doing tissue culture, minimizing chances of contaminating cultures.	Use ONLY metastatic cells.	Determine which experiment (experimental or spontaneous metastasis assay) is more appropriate.

Use only virus- and pathogen-free experimental animals from reputable vendors.	Do not assume that metastatic patterns will be identical using different host strains.	How many cells are to be injected? In what volume?	No bacterial or *Mycoplasma* contamination can be tolerated.	Use consistent conditions for preparing cells: confluence method of detachment growth medium passage number clone vs mixture pH of culture medium culture sterility logarithmic growth	Prior to study, do statistical power calculations to determine number of animals needed.
Obtain written reports. Routinely screen sentinel animals in-house to assure that no infections have occurred during an experiment.		Minimize embolization.			
Personnel who work with animals should avoid contact with non-research animals to avoid inadvertent cross-contamination.	Do not assume that more immunosuppressed animals will yield more metastases than immunocompetent hosts.	If using chimeric grafts, be careful to control for wound healing and surgical injury.	Minimal use of antibiotics and anti-fungal agents.	Minimize time from preparation of cells to injection.	Identify experimental model(s) that will most appropriately allow testing of hypothesis.
Lab personnel should monitor animals daily and more often as tumor burden increases.	Animals should be age-matched and sex-matched within experiments and between experiments.	Use only one injection site per animal.	Routinely screen cultures for contamination.	Minimize % dead cells in the inoculum (<10%).	Determine whether primary tumor remains or is to be removed.
Closely monitor food and water sources (maintain chlorine content <15 ppm).	Animals should come from same source (vendor) whenever possible.	Is anesthesia required to do the injection? If so, does the method influence metastatic potential?		When using tissues, prepare homogenous, sterile mixture.	
Minimize animal stress.		Do not pass cells through a needle under negative pressure.			

that 'confluence is an agent that promotes the progressive acquisition of the focus forming phenotype in clones of cells within NIH-3T3 populations. (a) even in cultures which have been confluent more than 2 wk without making foci, some have been affected by the confluent state and will make foci if replated and allowed to grow into new saturated cultures. (b) confluence causes foci to arise more frequently, compared to cells which has never been exposed to confluence; (c) the number and density of foci arising from replated confluent cells increase with the length of time spent in the confluent state; (d) confluence does not impact ... cells equally.' Likewise, Suzuki *et al.* [334] and Grdina *et al.* [335] previously showed a correlation between stage of cell cycle and lung colonization by a murine fibrosarcoma. To test whether differences in cell cycle distribution could explain variability in metastasis, (i.e. cells entering G_0/G_1), confluence was defined in our studies as the maximum number of cells per unit surface area prior to entering plateau phase. Thus, cell cycle distribution does not account for metastasis fluctuations resulting from different confluence levels in our experiments.

Our results are consistent with previously described alterations of metastasis-associated phenotypes as a function of cell density in tissue culture. Rizzino *et al.* [336] showed that binding to TGFβ was different depending upon confluence of the cell culture. Kaufmann *et al.* found that response to EGF was also different, depending upon the level of confluence [337]. Measurement of MMP2 and MMP9 in A431 epidermoid carcinoma cells was compared in sparse and confluent cultures. In sparse cultures, treatment of cells with phorbol myristate acetate or TGFβ induced low levels of MMP9; however, only TGFβ caused an effect in confluent cells. Neither treatment altered MMP2 levels. Interestingly, only cells at the edge of colonies express MMP9 when immunostained. This is analogous to the finding that proteinase secretion occurs predominately at the invasive edge of tumors [338]. Expression of basic fibroblast growth factor in a human renal cell carcinoma line is inversely proportional to confluence [339]. Since exogenous growth factors or autocrine response to growth factor signaling can change metastatic potential [340,341], altering the levels of secretion and/or response capability with confluence may be partially responsible for differences in metastatic potential.

Expression of surface MHC class I antigens, on the other hand, is 2- to 6-fold higher as cells become confluent [342] and release of soluble ICAM-1 changes with confluence [343]. Since MHC and ICAM-1 have been correlated to metastatic potential for some tumor types, modulation of expression in response to changes in confluence may involve these molecules. This has not been tested.

Two other melanoma cell lines, '26' and '37', have high and low rates of implantation, respectively. If sparse cultures of these cells were injected, the differential between the cells' lung colonization potential was nearly 8-fold. However, the difference decreased to 3-fold if confluent cells were used for metastatic potential [344]. Not only does this experiment show changes associated with confluence, it highlights the heterogeneity between cells in response to confluence. Likewise, Harris *et al.* [345] showed that metastatic potential of B16-F1 cells could be modulated by level of confluence at the time of cell preparation.

Size and shape change to varying extents during cell culture [334,346,347]. Raz and Ben Ze'ev [348,349] showed that cell shape can determine metastatic potential. Raz showed that altering the substratum by treatment with poly-HEMA (poly-(hydroxyethyl-methacrylate) transiently affected metastatic potential of B16-F1 cells [350]. Besides the inherent interest that metastasis could change according to cell shape, these experiments reveal the possibility that conditions at which cells are grown can alter metastasis.

Recently, Martínez-Zaguilán *et al.* [351] showed that invasiveness and motility of human melanoma cells is higher at pH 6.8 than pH 7.4. Even though buffered, acidity of culture media can fluctuate by 0.6 easily. This is particularly true if the buffering agent is sodium bicarbonate and incubator doors have been frequently entered immediately prior to cell preparation for injection.

Finally, Preziosi *et al.* [352] showed that the growth medium on which tumor cells are grown can profoundly influence differentiation and metastatic potential. B16-F10, -BL6 and -F1 cells were grown in either Dulbecco's modified Eagle's medium (DMEM) or RPMI-1640. Both media contain high levels of vitamins and trace minerals and, ostensibly, should not be limited in essential nutrients. In DMEM, the cells all had higher cAMP levels, greater tyrosinase activity, greater γ-glutamyl transferase and melanin production, as well as increased propensity for dendrite formation. The cells grown in DMEM also formed nine times as many lung metastases than equivalent cells grown in RPMI-1640. This experiment demonstrates that something as 'trivial' as changing the growth medium in which cells are grown can affect metastatic potential.

Taken together, these data clearly show that cell culture conditions can significantly alter the metastatic potential of cells. At minimum, 'trivial' changes have the potential to influence metastasis-associated phenotypes. As a result, conditions for culturing and preparing cells for injection must be carefully reported and followed precisely.

Concluding remarks

Metastasis is a complex problem. Models to study a complicated problem are correspondingly intricate. As a result, diligence is required in both experimental design and execution. The litany of considerations described above could appear daunting. But they should not discourage. Rather, they should be reminders of the complexities involved. But from complexity, two simple underlying reminders emerge. First, choose the right model and use it correctly. Second, until proven otherwise, assume that no experimental condition can be changed without affecting tumor cell behavior.

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Advances in Brief

Suppression of Metastasis in Human Breast Carcinoma MDA-MB-435 Cells after Transfection with the Metastasis Suppressor Gene, *KiSS-1*[1]

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Abstract

Based on the observation that chromosome 1q deletions are not infrequent in late-stage human breast carcinomas, we tested whether the recently discovered human melanoma metastasis suppressor gene, *KiSS-1*, which maps to chromosome 1q32-q41, could suppress metastasis of the human breast carcinoma cell line MDA-MB-435. Parental, vector-only transfectants and *KiSS-1* transfectant clones were injected into the mammary fat pads of athymic nude mice and assessed for tumor growth and spontaneous metastasis to regional lymph nodes and lungs. Expression of *KiSS-1* reduced metastatic potential by 95% compared to control cells but did not suppress tumorigenicity. Metastasis suppression correlated with a decreased clonogenicity in soft (0.3%) and hard (0.9%) agar. Although the overall rate of cell adhesion to extracellular matrix components was unaffected, *KiSS-1* transfectants spread on immobilized type-IV collagen more rapidly than did control populations. Invasion and motility were unaffected by *KiSS-1*. Based on the predicted structure of the KiSS-1 protein, our results imply a mechanism whereby KiSS-1 regulates events downstream of cell-matrix adhesion, perhaps involving cytoskeletal reorganization. In addition to its already described role in melanoma, our results show that *KiSS-1* also functions as a metastasis suppressor gene in at least some human breast cancers.

Introduction

KiSS-1 was identified as a human melanoma metastasis suppressor gene using subtractive hybridization between the metastatic human melanoma cell line C8161 and nonmetastatic variants generated after microcell-mediated transfer of chromosome 6 into C8161 (1). Transfection of *KiSS-1* into metastatic human melanoma cell lines C8161 and MelJuSo suppressed metastasis in athymic nude mice by 50–95% (1, 2). The *KiSS-1* gene maps to chromosome 1 bands q32–q41 and predicts a hydrophilic, 164-amino acid protein with a proline-rich domain suggestive of an SH3 ligand and a putative protein kinase C-α phosphorylation site. These domains within KiSS-1 suggest that KiSS-1 could be involved in the signal transduction pathway.

The purpose of this study was to determine whether *KiSS-1* might function as a metastasis suppressor in another tumor type. Breast cancer is the most common cancer among women in North America and western Europe and is the second leading cause of female cancer deaths in the United States (3). Like most solid tumors, metastatic disease rather than the primary tumor itself is responsible for death. The rationale for studying *KiSS-1* in human breast cancer is based on studies showing loss of heterozygosity for regions of chromosome 1q (4–6). Involvement of chromosome 1q is not frequent in most cancers; therefore, the frequency of loss of heterozygosity suggested that a gene involved in breast cancer progression was encoded therein.

Here we report a test of that hypothesis using transfection of the metastatic human breast carcinoma cell line MDA-MB-435 with *KiSS-1* cDNA. Transfectants formed significantly fewer pulmonary and regional lymph node metastases than did control or neo-transfected clones. These results suggest that *KiSS-1* can function as a breast cancer metastasis suppressor gene and may indeed be involved in the progression of human breast cancer toward malignancy.

Materials and Methods

Cell Lines and Culture. MDA-MB-435 and MDA-MB-231 are estrogen receptor- and progesterone receptor-negative, metastatic, ductal breast carcinoma cell lines (7). Both cell lines form tumors when injected into the mammary fat pads of nude mice, and macroscopic metastases to lungs and regional lymph nodes can be identified 10–18 weeks postinoculation. However, MDA-MB-435 forms more metastases in a greater percentage of athymic nude mice than do the MDA-MB-231 cells. Therefore, the former were chosen for transfection studies. MDA-MB-435 cells were the generous gift of Dr. Janet Price (University of Texas M. D. Anderson Cancer Center, Houston, TX). MDA-MB-231 cultures were kindly provided by Dr. Robert Gillies (University of Arizona Cancer Center, Tucson, AZ).

Both human breast cancer cell lines were maintained in DMEM:F12 medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (cDMEM:F12; Atlanta Biologicals) and no antibiotics. The neomycin-resistant *KiSS-1* transfectants were maintained in cDMEM:F12 containing 500 μg/ml G418 (Life Technologies, Inc., Gaithersburg, MD). All cell lines were free of *Mycoplasma* sp. contamination as determined by a PCR-based test (PanVera, Madison, WI).

Transfection of *KiSS-1*. The construction of pcDNA3-*KiSS-1* expression vector was described previously (1). pcDNA3-*KiSS-1* vector was transfected into MDA-MB-435 cells using Lipofectin (Life Technologies, Inc.) according to the manufacturer's instructions. pcDNA3 vector only was also transfected as a control. G418-resistant clones were isolated by growth in selective medium. Uncloned, stable transfectant cells (MDA-MB-435-*KiSS-1* mix) were cloned by limiting dilution (MDA-MB-435-*KiSS-1* cl.1, MDA-MB-435-*KiSS-1* cl.2, MDA-MB-435-*KiSS-1* cl.3, and MDA-MB-435-*KiSS-1* cl.7).

Northern Blotting. RNA expression analysis was done as described previously. Total RNA from cell cultures and tumor tissues was isolated using a RNeasy Kit® (Qiagen, Chatsworth, CA). Poly(A)$^+$-enriched[3] mRNA was isolated using a Micro FastTrack kit® (Invitrogen, San Diego, CA). Total RNA (20 μg) or poly(A)$^+$-enriched RNA (2.5 μg) was size-fractionated in a 1% agarose gel containing 2.2 M formaldehyde. After transfer and UV cross-linking, the nylon membranes were probed with a full-length ^{32}P-labeled *KiSS-1* cDNA.

Migration and Adhesion Assays. Migration was measured by the wounding method as described previously (8). Briefly, breast carcinoma cells (4×10^5) were plated onto 6-well plates in triplicate and allowed to grow until 80% confluence. The monolayer was scraped with a Teflon-coated plastic cell scraper (Fisher Scientific, Pittsburgh, PA), and the detached cells were removed by washing three times with CMF-DPBS. The remaining adherent cells were incubated in DMEM:F12 containing 0.5% fetal bovine serum for 24–48 h. Photographs of the edge were used to compare the migration of cells into the exfoliated space.

Adhesion was measured in a 96-well plate assay. Briefly, wells were coated

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[3] The abbreviations used are: poly(A)$^+$, polyadenylate; CMF-DPBS, calcium- and magnesium-free Dulbecco's PBS.

with mouse laminin, fibronectin, or collagen type IV (Collaborative Biomedical Product, Bedford, MA) at a concentration of 5 $\mu g/cm^2$ for 1 h at room temperature. Murine adhesion molecules were used, because this is the environment onto which cells would have to adhere *in vivo*. The wells were then rinsed with CMF-DPBS before preblocking with a solution of DMEM:F12 containing 1% BSA for 1 h at 37°C. After removing the supernatant, cells (4×10^4) suspended in adhesion medium (DMEM:F12 supplemented with 0.5% BSA and 25 μM HEPES) were dispensed into each well, incubated at 37°C for varying times, and gently washed three times with CMF-DPBS. The remaining adherent cells were quantified by measuring acid-phosphatase (9). To semiquantify the spreading results, the percentage of spread/flattened cells per random high-power field were determined.

Photographs of cells at various times after placement onto the matrices were taken to compare spreading. Tissue culture plates (24 well) were coated with 5 $\mu g/cm^2$ mouse laminin, fibronectin, or collagen type IV as described above. Cells (2×10^5) were suspended in adhesion medium and dispensed into each well before incubation at 37°C for 3 h. Unattached cells were removed by washing with CMF-DPBS.

Clonogenic Soft Agar Assays. Colonization in soft agar was performed as described previously (10), with minor modifications. A layer of cDMEM:F12 with 0.5% agarose was set into 6-well plates. A suspension of cells (10^2-10^3) in 0.3% agarose was overlaid onto the basal layer. A similar experiment was performed using hard agar (0.9%) as an upper layer, because this parameter has been shown to be more closely related to metastatic efficiency than soft agar cloning (10). The number of colonies was determined 20–40 days after plating.

Metastasis Assays. Cells (10^6) were injected into the subaxillary mammary fat pads of 4–6-week-old female athymic nude mice (7–10 mice/group; Harlan Sprague Dawley, Madison, WI). Mice were maintained under the guidelines of the NIH and The Pennsylvania State University College of Medicine. All protocols were approved and monitored by the Institutional Animal Care and Use Committee. Food and water were provided *ad libitum*. When the mean tumor diameter (square root of the product of orthogonal measurements) reached 1.0–1.3 cm, primary tumors were surgically removed under Ketaset-Rompun anesthetic. Mice were then maintained for an additional 4 weeks to allow further growth of lung metastases. After euthanasia, all organs were checked for metastasis. Visible lung metastases were counted in fixed tissues (neutral-buffered formalin:Bouin's fixative, 5:1) with the aid of a dissecting microscope as described previously (11).

Statistical Analysis. The number of lung metastases in *KiSS-1* transfectants and control cells (parental and vector-only transfectants) was compared using a Kruskal-Wallis ANOVA of ranks procedure. The percentage of spread/flattened cells on type-IV collagen matrix was compared using the Kruskal-Wallis ANOVA of ranks procedure. All calculations were performed using SigmaStat statistical analysis software (Jandel Scientific, San Rafael, CA). Statistical significance was defined as $P \leq 0.05$.

Results

Expression of the *KiSS-1* transcript in MDA-MB-435 and MDA-MB-231 cells was measured by Northern blot (Fig. 1*A*). MDA-MB-435 cells are reportedly less invasive *in vitro* than MDA-MB-231 cells but are significantly more metastatic (7). MDA-MB-435 cells do not express the *KiSS-1* transcript (1.0 kb), but MDA-MB-231 cells do. Based on their relative *KiSS-1* expression and metastatic potentials, MDA-MB-435 cells were chosen for subsequent studies.

Full-length KiSS-1 cDNA was subcloned into the pcDNA3 constitutive expression vector and transfected into MDA-MB-435 cells. Concomitantly, vector without insert was transfected as a negative control. Several single-cell clones were randomly selected after limiting dilution, and the expression of *KiSS-1* was confirmed by Northern blot (Fig. 1*B*). Transfectants and matched controls were inoculated into the subaxillary mammary fad pads of 4–6-week-old female athymic nude mice. Tumors were measured weekly thereafter to assess the growth rate. Once the mean tumor diameter reached ≈1 cm, tumors were surgically removed. One month later, mice were euthanized, and metastases were quantified. Primary tumor removal was done to minimize the effect of different growth rates for clonal populations and to allow micrometastases time to develop into macroscopic lesions. Local tumors were removed from MDA-MB-435-, MDA-MB-435-*KiSS-1* mix-, MDA-MB-435-*KiSS-1* cl.1-, MDA-MB-435-*KiSS-1* cl.2-, MDA-MB-435-*KiSS-1* cl.3-, and MDA-MB-435-*KiSS-1* cl.7-injected mice 49, 49, 69, 106, 106, and 53 days after injection, respectively.

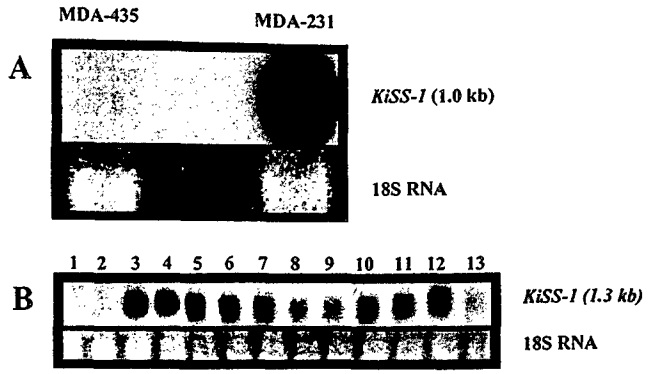

Fig. 1. Northern blot analysis of MDA-MB-231, MDA-MB-435, and *KiSS-1* transfectants for *KiSS-1* mRNA expression. *A*, *KiSS-1* expression in human breast carcinoma cell lines MDA-MB-231 and MDA-MB-435. Poly(A)$^+$-enriched RNA was prepared from exponentially growing MDA-MB-231 and MDA-MB-435 cells. Agarose-formaldehyde gels were loaded with 2.5 μg of RNA/lane and hybridized with radiolabeled full-length *KiSS-1* probe. 18S RNA is shown as a loading control after the gel was stained with ethidium bromide. *B*, *KiSS-1* expression in *KiSS-1*-transfected MDA-MB-435 cell lines. Total RNA was prepared from cultured cells and tumor tissue. Gels were loaded with 20 μg RNA/lane, transferred to nylon membranes, and hybridized with radiolabeled full-length *KiSS-1* probe. 18S RNA is shown as a loading control after the gel was stained with ethidium bromide. *Lane 1*, MDA-MB-435 tumor; *Lane 2*, MDA-MB-435 vector-only transfectant tumor; *Lane 3*, MDA-MB-435-*KiSS-1* mix cells; *Lane 4*, MDA-MB-435-*KiSS-1* cl.2 cells; *Lane 5*, MDA-MB-435-*KiSS-1* mix cells; *Lane 6*, MDA-MB-435-*KiSS-1* cl.1 cells; *Lane 7*, MDA-MB-435-*KiSS-1* cl.1 tumor; *Lane 8*, MDA-MB-435-*KiSS-1* cl.2 cells; *Lane 9*, MDA-MB-435-*KiSS-1* cl.2 tumor; *Lane 10*, MDA-MB-435-*KiSS-1* cl.3 cells; *Lane 11*, MDA-MB-435-*KiSS-1* cl.3 tumor; *Lane 12*, MDA-MB-435-*KiSS-1* cl.7 cells; *Lane 13*, MDA-MB-435-*KiSS-1* cl.7 tumor.

Examination of H&E-stained sections of parental MDA-MB-435 and *KiSS-1* transfectant tumors was also done. Both tumor types exhibited characteristics of poorly differentiated invasive adenocarcinoma (data not shown), but we found no consistent histological properties that distinguished *KiSS-1* transfectants from parental cells.

Tumorigenic and metastatic properties are depicted in Table 1 and Fig. 2. All cells formed tumors; however, the number of macroscopic lung metastases in animals injected with the *KiSS-1* transfectants was significantly ($P < 0.05$) decreased. The *KiSS-1* transcript (1.3 kb) was still expressed in tumor tissues (Fig. 1*B*), showing that tumor growth was not due to loss of the vector. Parental MDA-MB-435 cells formed an average of 26 lung metastases/mouse. Two vector-only transfectants formed 9 and 18 lung metastases, respectively. The *KiSS-1* transfectants formed between 1 and 5 lung metastases/mouse. These metastases were extremely small compared to those found in the parental cells or in vector-only transfectants. The presence of additional microscopic metastases in random lung sections was not observed by H&E staining (data not shown). Of parental and neo transfectants, only 1 mouse had no metastasis (1 of 24 mice, 4.2%), whereas 12 of 24 mice (50%) developed >10 lung metastases. In *KiSS-1* transfectants, 12 of 39 mice (30.8%) developed no lung metastases, and only 2 of 39 mice (5.1%) had >10 metastases/animal. In all *KiSS-1* transfectants, metastasis to lung was suppressed by at least 50%. Likewise, the incidence of regional lymph node metastasis was reduced. These results clearly demonstrate that the expression of *KiSS-1* in human MDA-MB-435 breast carcinoma cells significantly suppresses metastatic ability in athymic nude mice.

The mechanism by which *KiSS-1* suppresses metastasis in melanoma cells has still not been determined. Neither adhesion to extracellular matrices nor invasiveness *in vivo* or through reconstituted basement membranes using *in vitro* assays is altered in melanoma *KiSS-1* transfectants. These findings, however, could not preclude the action of *KiSS-1* at these steps in breast cancer metastasis. *KiSS-1* transfectants are

Table 1 *KiSS-1 suppresses metastasis of MDA-MB-435 cells from the mammary fat pad in athymic nude mice*[a]

Cell line	Tumorigenicity	Lung metastases			Lymph node metastases
		Incidence[b]	Mean ± SEM	Median (range)	Incidence[b]
MDA-MB-435	8/8	8/8	26 ± 18	8.5 (3–153)	5/8
Vector 1[c]	8/8	8/8	18 ± 8	10.5 (3–71)	4/8
Vector 2	8/8	7/8	9 ± 4	5.5 (0–35)	5/8
*KiSS-1*mix	8/8	4/8	1 ± 1	0.5 (0–6)[d]	2/8
KiSS-1.cl.1	8/8	6/8	5 ± 2	2.5 (0–21)[d]	0/8
KiSS-1.cl.2	8/8	6/8	2 ± 1	1.5 (0–7)[d]	0/8
KiSS-1.cl.3	8/8	4/8	1 ± 0.4	0.5 (0–2)[d]	0/8
KiSS-1.cl.7	7/7	7/7	3 ± 1	3.0 (1–10)[d]	0/8

[a] Cells (10^6) were injected into the subaxillary mammary fat pads of 4–6-week-old female athymic nude mice. When the mean tumor diameter reached 1.0–1.3 cm, primary tumors were surgically removed. Metastases were quantified 4 weeks later.
[b] Number of mice with metastases/total number of mice injected.
[c] The pcDNA3 vector without an insert. *KiSS-1*mix is the uncloned population of pcDNA3-*KiSS-1* transfectants.
[d] Significantly different ($P < 0.05$) from parental cells or vector-only transfectants by Kruskal-Wallis ANOVA.

as invasive as parental MDA-MB-435 cells *in vivo* (data not shown); therefore, *in vitro* assays were not done. Likewise, significant differences in migration were not observed (data not shown).

Although the kinetics of adhesion to tissue culture plates coated with murine collagen type IV, laminin, or fibronectin were unchanged in *KiSS-1* transfectants (*i.e.*, the number of cells attached throughout the adhesion assay was identical to that of untransfected controls; data not shown), the rate of spreading (*i.e.*, flattened morphology) on type-IV collagen was different (Fig. 3). Parental and vector-only transfectant cells were still predominantly rounded 3 h after plating onto type-IV collagen. Less than 5% of cells exhibited a flattened morphology. In contrast, more than 90% of *KiSS-1* transfectants had spread significantly in the same time.

KiSS-1 expression also correlated with a significantly reduced ability to form colonies in both soft (0.3%) or hard (0.9%) agar (Fig. 4). This parameter was examined because of published reports demonstrating a correlation between clonogenicity on hard and/or soft agar and metastatic propensity (10).

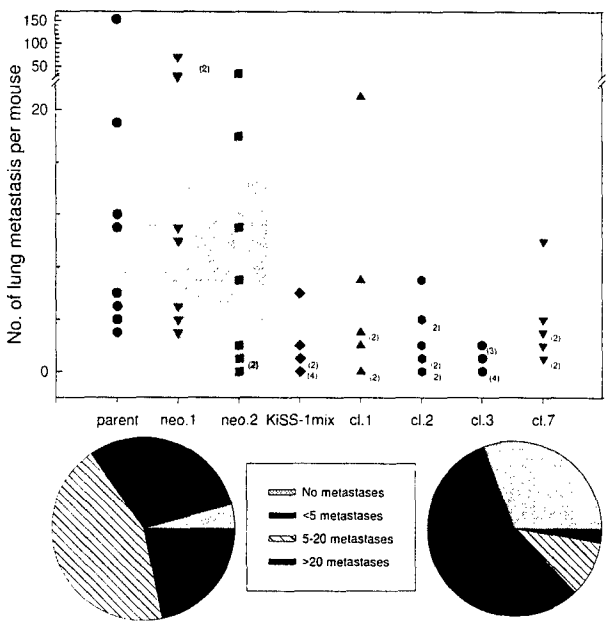

Fig. 2. Formation of lung metastases transfectants is decreased in *KiSS-1* transfectants. *Upper panel*, the number of metastases/mouse injected with variants of MDA-MB-435 (parent) cells. *Each symbol*, one mouse. *Numbers to the right of symbols*, more than one mouse had a particular number of metastases. *Lower panel*, the distribution of lung metastases number in control (*shaded* in *upper panel*) *versus KiSS-1* transfectants. Cells (10^6) were injected into the mammary fat pads of 4–6-week-old female athymic nude mice. When the mean tumor diameter reached ≈1.0 cm, primary tumors were removed, and the mice were maintained for 4 weeks to allow further growth of lung metastases.

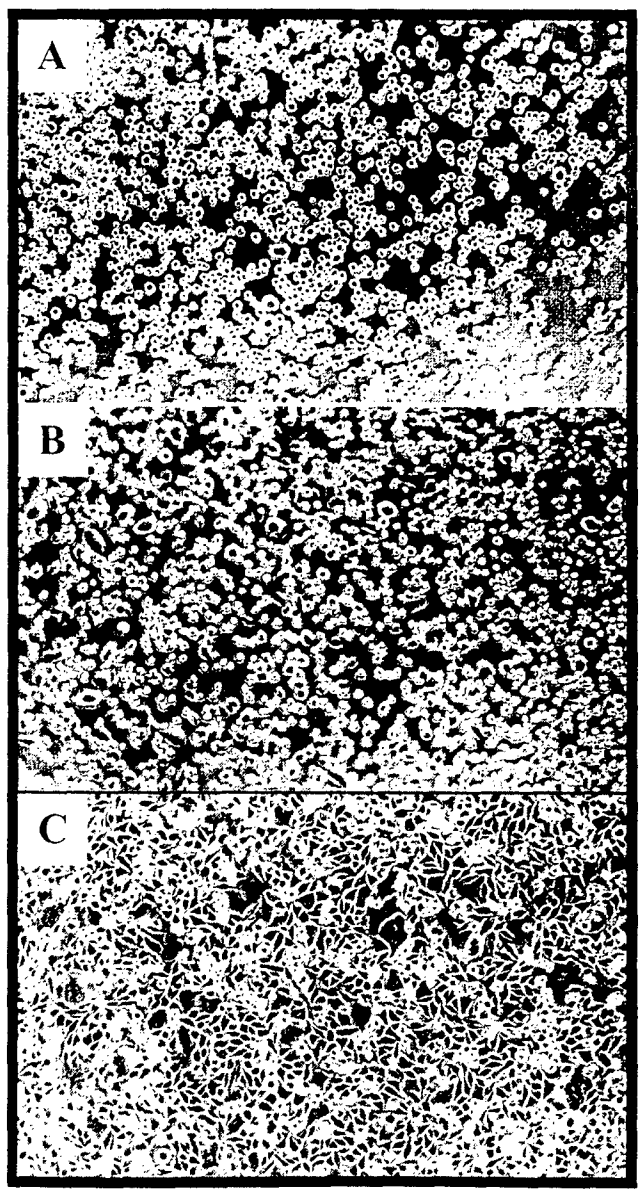

Fig. 3. Differential spreading of parental MDA-MB-435 (*A*), vector-only transfectant (*B*), and *KiSS-1*-transfected (*C*) cells on immobilized type-IV collagen. Exponentially growing cells were plated onto type-IV collagen-coated tissue culture plates and photographed 3 h later. Note the increased number of spreading cells in *C* compared to *A* and *B*. Counting of spread/flattened cells within random high-power fields was done to compare parental, neo-transfected, and *KiSS-1*-transfected cells. Spread/flattened parental and neo-transfected cells comprise <5% of the population, whereas >90% of *KiSS-1* transfectants are spread 3 h after seeding onto type-IV collagen matrix. Magnification, ×100.

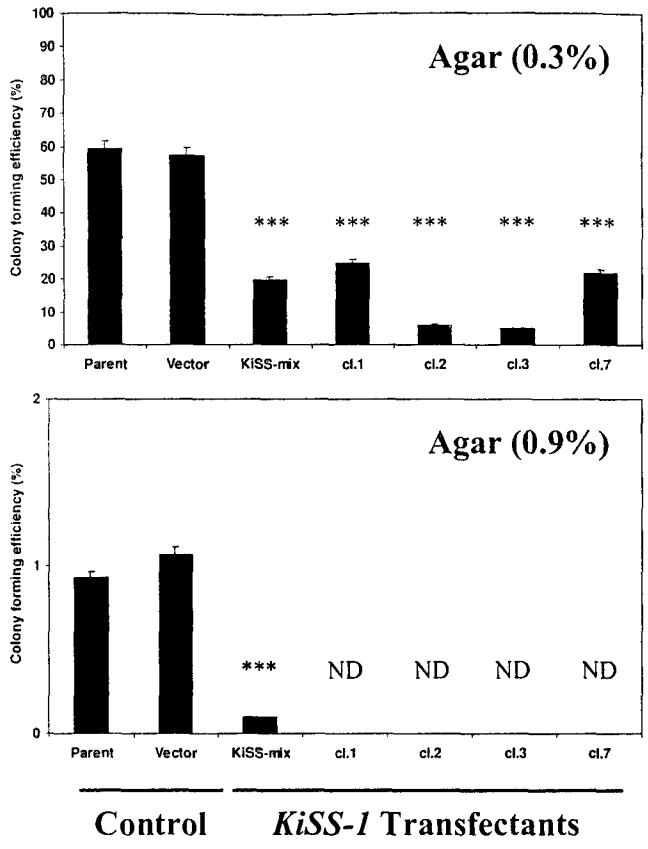

Fig. 4. Clonogenicity of MDA-MB-435 variants in soft (0.3%) and hard (0.9%) agar. *Values*, mean plating efficiency ± SE *KiSS-1* transfectants form significantly (***, $P < 0.05$) fewer colonies than parental or vector-only transfectants as determined by one-way ANOVA. *ND*, not tested.

Discussion

Based on the relatively low frequency of chromosome 1q alterations in most human neoplasms except breast tumors (12) and the map location of the recently described human melanoma metastasis suppressor gene *KiSS-1*, we hypothesized that the restoration of expression of *KiSS-1* into a metastatic human breast carcinoma cell line should suppress metastasis. Our data clearly show that *KiSS-1* can function as a metastasis suppressor in the MDA-MB-435 breast carcinoma. Parental MDA-MB-435 cells and tumor tissue did not express the *KiSS-1* transcript. Tumor tissue collected from mice injected with *KiSS-1* transfectants continued to express *KiSS-1* mRNA. The lung metastases from *KiSS-1* transfectants were not visible before fixing with Bouin's fixative and examination under a dissecting microscope; therefore, assessment of *KiSS-1* was not determined. Based on past experience with these cells and this vector, the likelihood that the vector was spontaneously lost is low, but this possibility cannot be excluded.

In preliminary experiments, the *KiSS-1* transcript has been routinely detected in normal breast tissue adjoining biopsy specimens (data not shown). This suggests that *KiSS-1* may inversely correlate with the progression of breast tumors. It will be necessary to extend our findings to a larger panel of cell lines or to tumors with different metastatic potentials to firmly establish a role for *KiSS-1* in breast tumor progression. Unfortunately, there are few human breast carcinoma cell lines that reproducibly metastasize in athymic nude mice.

Although *KiSS-1* encodes a protein with a putative SH3 ligand, its mechanism of action remains unknown. SH3 domains are frequently found in many proteins that mediate protein-protein interactions important for signaling and cytoskeletal organization (13, 14). Thus, we have focused our attention on steps in the metastatic cascade with known signaling and/or cytoskeletal involvement.

KiSS-1 expression in melanoma does not correlate with decreased invasiveness, nor does it correlate with the ability to adhere to basement membrane components. These conclusions were corroborated in the MDA-MB-435 breast carcinoma model. The ultimate levels of adhesion to extracellular matrix components did not change; however, *KiSS-1* transfectants did spread on type-IV collagen more quickly than did control cells. The mechanism underlying this change is not known, but this is the first evidence that *KiSS-1* may be involved, at least tangentially, in cellular cytoskeletal organization.

Previous reports suggested that clonogenicity in agar, especially in hard agar, correlated with the metastatic potential of MDA-MB-435 cells (10). Our results generally agree with their findings. *KiSS-1* transfectants formed significantly fewer colonies than did control cells. Again, this finding is consistent with altered cell structure in *KiSS-1* transfectants.

In summary, transfection of the human melanoma metastasis suppressor gene *KiSS-1* into MDA-MB-435 human breast carcinoma cells significantly suppressed metastasis in athymic nude mice. This extends to three the number of cell lines transfected with *KiSS-1* that show marked suppression of metastasis without alteration of tumor-forming ability. The results presented in this paper show that *KiSS-1* probably functions to control metastasis in a pathway shared by at least two tumor cell types of distinct embryonic origin, breast cancer and melanoma. Our results also imply that *KiSS-1* might be a gene mapping to chromosome 1q, which was previously implicated in breast cancer progression by loss-of-heterozygosity studies, and although they do not prove a role for *KiSS-1* in cytoskeletal organization, differential spreading on type-IV collagen and anchorage-independent growth are consistent with the predicted function of *KiSS-1* as an SH3 ligand.

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Advances in Brief

Suppression of MDA-MB-435 Breast Carcinoma Cell Metastasis following the Introduction of Human Chromosome 11[1]

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Abstract

To determine the relevance of genetic information on chromosome 11 in the development of metastatic breast tumors, we introduced a normal human chromosome 11 into the highly metastatic MDA-MB-435 breast carcinoma cell line via the microcell-mediated chromosome transfer technique. Although the MDA-MB-435 recipient cell line and four randomly selected microcell hybrid clones remained tumorigenic in nude mice, the hybrids were >95% suppressed for metastasis to lung and regional lymph nodes ($P < 0.01$). We also tested whether chromosome 6 harbors a metastasis-suppressor gene for breast cancer as observed previously for human melanoma. Grouped together, the four neo6 microcell hybrids had no statistically significant reduction in the incidence or number of lung or lymph node metastases compared to the weakly metastatic, subcloned parent cell line, MDA-MB-435.7. Expression of *nm23-H1* (NME1), a known metastasis-suppressor gene in this breast cancer cell line, did not correlate with metastasis suppression in the microcell hybrids. These results further demonstrate that control of metastasis is molecularly distinct from tumorigenic potential. They also indicate that chromosome 11 encodes a metastasis-suppressor gene for human breast cancer.

Introduction

Delineating the molecular events controlling development of breast cancer can increase our knowledge of how all tumor cells progress toward malignancy. In breast cancer, as in most solid tumors, it is usually the metastatic disease rather than the primary tumor that causes death. Several studies have shown that metastasis is a molecular event distinct from initial tumor formation and that cells progress to metastatic capability after accumulating several genetic defects (1–4). Thus, characterization of the later molecular events in aggressive tumor cells can have several potential benefits. These include understanding the interplay between cancer suppressor genes and cell cycle genes like *p53*, the contributions of genetic susceptibility, and the impact of environmental factors on the development of metastasis. Over 46,000 women will die from breast cancer this year (5). Therefore, knowledge of genetic loci whose loss or inactivation contributes to metastasis development can help with decisions of treatment and prognosis of this disease.

Loss of heterozygosity studies have shown that regions on chromosomes 6 and 11 are frequently lost or mutated during the development of breast tumors (6–12). These genetic alterations suggest the presence of suppressor genes, the inactivation of which allows tumor initiation and metastases. Many studies have demonstrated that the introduction of normal chromosomes into malignant cell lines may restore gene function and reverse the transformed phenotype. In fact, monochromosomal transfer of human chromosome 11 suppressed the tumorigenic phenotype of several tumor lines (13, 14), including the MCF-7 human breast cancer cell line (15). The metastatic phenotype can be suppressed by transfer of chromosome 6 into melanoma cells (4) and by chromosome 17 transfer into rat prostate and rat mammary carcinoma cells (3). Yet suppressor gene function is often tumor-type specific, since transfer of a chromosome 11 fragment (pter–q14) via MMCT[4] did not inhibit metastasis in rat mammary cells but did suppress metastasis in rat prostate cells (3).

To determine if a gene on chromosome 6 or 11 might alter the tumorigenic or metastatic phenotype of a human breast carcinoma cell line, we introduced neo-tagged chromosomes into the MDA-MB-435 breast cancer cell line. Introduction of chromosome 6 had little influence on the cells. However, chromosome 11 microcell hybrids showed significantly reduced metastatic potential. Our results indicate that human chromosome 11 harbors a metastasis-suppressor gene for human breast cancer.

Materials and Methods

Cell Lines. The MDA-MB-435 (MDA 435) cell line is an ER- and PR-negative, metastatic, ductal human breast carcinoma cell line derived from a 31-year-old female (16). It has a heterogeneous chromosome complement of between 50 and 58 chromosomes, including several derivative marker chromosomes. The cell line readily forms tumors when injected into the mammary fat pads of nude mice (17), and macroscopic metastases to lungs and regional lymph nodes can be identified 10–12 weeks postinoculation. This pattern closely parallels clinical observations. The MDA-MB-435 cell line and a subclone, MDA-MB-435.7, were used as the recipient cell populations in MMCT experiments. Chromosome donor lines were immortalized mouse A9 cell lines carrying single human chromosomes tagged with the selectable neomycin resistance gene; the MCH556.1c5 donor line contains human chromosome 11 (18), and the MCH262A1.D6 donor line contains human chromosome 6 (19). The recipient cell lines were maintained in RPMI or DMEM/Ham's F-12 media supplemented with 10% FBS and no antibiotics. The donor cell lines and all microcell hybrid cell lines were maintained in media additionally supplemented with 600 μg/ml G418 (Sigma). All cells were free of *Mycoplasma spp.* contamination, as determined by PCR (Stratagene) and by Gen-probe Rapid Detection (San Diego, CA).

MMCT. Microcell hybrids were generated as described previously (13) or by the technique of Stubblefield and Pershouse (20). Neo-resistant microcell hybrids were selected in growth medium containing 600 μg/ml G418. Individual clones were isolated and expanded for characterization. Hybrids derived from microcell fusion with chromosomes 6 and 11 were designated neo6/435.7 and neo11/435, respectively.

Cytogenetic Analysis. Karyotypes of the recipient cell lines and microcell hybrids are based on the examination of at least 15 metaphase spreads that were banded by the trypsin-Giemsa G-band method (21). The integrity of the

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[4] The abbreviations used are: MMCT, microcell-mediated chromosome transfer; ER, estrogen receptor; PR, progesterone receptor; FISH, fluorescence *in situ* hybridization; RMP, relative metastatic potential.

human chromosome in the donor cell lines was verified by the G-11 differential staining technique (22). For ease of identification, chromosomes 6 and 11 were sometimes visualized by FISH whole chromosome painting (23). Briefly, DNA from each donor cell line was amplified with the following human alu primers: A1, L1, alu278, and pDJ67 (24). The amplified products were biotinylated by random-primed labeling (Vector Labs) and hybridized to microscope slides containing metaphase spreads of the microcell hybrids. Chromosomes could be detected following an incubation with FITC avidin, although re-amplification of the signal with biotinylated anti-avidin followed by a second incubation in FITC-avidin was necessary for strong signal.

PCR Analysis of Polymorphic Dinucleotide Repeat Sequences. The presence of the introduced chromosome in each microcell hybrid was verified by amplification of polymorphic dinucleotide repeats from the p and q arms of chromosomes 6 and 11 (Fig. 1). DNA isolated from the recipient cell line and microcell hybrids was amplified with primers end-labeled with [γ-^{32}P]dATP, as described previously (25). Radioactive amplicons were separated on a 6% polyacrylamide sequencing gel and detected after overnight exposure to X-ray film. At least three informative loci were needed to confirm that the microcell hybrid cell lines contained the introduced chromosome. In one case, a mini-satellite locus (primer pMS51) was used that amplified products large enough to be distinguished on an ethidium bromide-stained, 1% agarose gel (6).

Spontaneous Metastasis Assay. Cells (5×10^5 to 1×10^7) were injected into the subaxillary mammary fat pads of three to 5-week-old female athymic mice (Harlan Sprague-Dawley or NCI). Mice were killed when the mean tumor diameter (square root of the product of orthogonal measurements) reached 1.5–2.0 cm or when mice were moribund. All organs were checked for metastases. Visible lung metastases were counted in Bouins' fixed tissues, as described previously (26). Tumors isolated from mice receiving neo6/435.7 and neo11/435 injections retained the introduced chromosome, as assayed by karyotype and by PCR using anonymous $(CA)^n$ polymorphisms. Metastases from neo11/435 microcell hybrids were too small for detection without a microscope. Attempts to culture revertants in G418-containing media yielded no growth.

Northern Blot Analysis. Poly(A)+ enriched RNA (2.5 μg) was purified using the Fas-track mRNA Isolation Kit (Invitrogen, San Diego, CA) and was size-fractionated using a 1% agarose, 1 M formaldehyde gel. After transfer and cross-linking to a nylon membrane (Schleicher & Schuell), RNA was probed with a 0.8-kb [^{32}P]*Bam*HI fragment of *nm23-H1* at 42°C. The same blot was stripped and reprobed for β-actin as a loading control.

pnm23-H1 was generously provided by Dr. Patricia S. Steeg (National Cancer Institute). DNA fragments used for probes were digested and separated on a 0.8% agarose gel, excised, prepared with PCR Prep (Promega, Madison, WI), and labeled by random priming with [α-^{32}P]dCTP (Amersham).

A.

CELL LINE	CYTOGENETICS	POLYMORPHIC MARKERS			
	CHROMOSOME 11 ANALYSIS	pMS51[1] (11q13)	D11S419 (11p14-15)	ApoC3 (11q23)	D11S1367 (q13.1-22.3)
MCH556.1c5	one cs 11	A	A	A	A
MDA-MB-435	one cs 11 + der(11) + t(11q;13q)	B	B,C	B,C	B,C
neo11/435.A3	two cs 11 + der(11)	A,B	A, C	A, C	A, C
neo11/435.B1	two cs 11 + der(11) + t(11q;13q)	A,B	A,B,C	A,B,C	A,B,C
neo11/435.D1	one cs 11 + der(11) + t(11q;13q)	A,B	A,B,C	A,B,C	A,B,C
neo11/435.E1	two cs 11 + der(11) + t(11q;13q)[2]	A,B	A,B,C	A,B,C	A,B,C
	CHROMOSOME 6 ANALYSIS	D6S257[3] (6cen)	D6S309 (6pter)	D6S278 (6q14)	D6S279 (6q23)
MCH262A1.D6	one cs 6	A	A	A	A
MDA-MB-435.7	one cs 6 + der(6) + t(6;?)	A,B	B,C	B,C	B,C
neo6/435.7.A1	two cs 6 + der(6) + t(6;?)	A,A,B	A,B,C	A,B,C	A,B,C
neo6/435.7.C1	two cs 6 + der(6) + t(6;?)	A,A,B	A,B,C	A,B,C	A,B,C
neo6/435.7.E2	two cs 6 + der(6) + t(6;?)	A,A,B	A,B,C	A,B,C	A,B,C
neo6/435.7.G1	two cs 6 + der(6) + t(6;?)[2]	A,A,B	A,B,C	A,B,C	A,B,C

B.

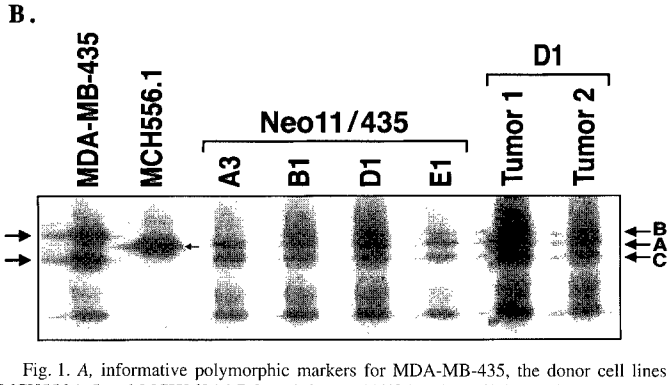

Fig. 1. *A*, informative polymorphic markers for MDA-MB-435, the donor cell lines MCH556.1c5 and MCH262A1.D6, and the neo11/435 and neo6/435.7 microcell hybrid clones. Primers listed flank regions of dinucleotide repeats and are used to specifically verify the introduction of chromosomes 6 and 11. The following additional chromosome 11 polymorphic markers were used with results similar to those shown: *D11S1384, D11S1333,* and *D11S1340*. The cytogenetics indicate the numbers of specific chromosomes present in the parents and microcell hybrids. *1*, allele B was amplified in both copies of chromosome 11 in the MDA-MB-435 cell line; therefore, there is no third allele C. *2*, cytogenetic analysis revealed that 30% of the neo11/435.E1 population and 50% of the neo6/435.7.G1 population were polyploid. *3*, both donor and recipient cell lines amplified for allele A; however, the hybrids contained twice the amount of this allele, as determined by densitometry (data not shown). *B*, a representative autoradiograph of a dinucleotide repeat polymorphism for chromosome 11. *Arrows*, alleles A, B, and C for the ApoC3 primer set.

Results

Characterization of Cell Lines. To examine the chromosome complement of the MDA-MB-435 breast cancer cells, metaphase spreads were analyzed by trypsin-Giemsa G-banding. In addition, FISH painting was used to identify the intact copies of chromosome 11 and translocated sections. The MDA-MB-435 cell line is aneuploid with a modal number of 56 chromosomes (range, 50–58). The cell population is heterogeneous, as the chromosome complement differed between the recipient cells, subclones of the recipient cells, and some of the microcell hybrids. As shown in Fig. 2, MDA-MB-435 contains one cytogenetically normal chromosome 11, one or two derivative copies of chromosome 11, and usually (>90%) a translocated chromosome t(11q;13q).

Microcell hybrids containing an introduced human chromosome 11 were generated by fusion of the MDA-MB-435 cells with neo-tagged chromosome 11 microcells prepared from MCH556.1c5. Hybrids were selected in media supplemented with G418. Four random microcell hybrid lines, designated neo11/435.A3, neo11/435.B1, neo11/435.D1, and neo11/435.E1, were chosen for further analysis. To confirm the successful introduction of the neo-11 chromosome, DNA from the recipient, donor, and microcell hybrid lines was amplified with several primers that flank polymorphic regions of dinucleotide repeats representing short- and long-arm sequences. As shown in Fig. 1, each neo11/435 hybrid generated PCR alleles specific for both the MCH556.1c5 donor and the MDA-MB-435 recipient line. Cytogenetic analysis also verified the presence of the additional chromosome 11 in three of the four microcell hybrids. The neo11/435.B1 hybrid (Fig. 2) contained two normal chromosomes 11, a derivative chromosome 11, and the t(11q;13q) chromosome. The neo11/435.E1 hybrid also contained an extra normal chromosome 11, although approximately 30% of this cell population was polyploid (Fig. 1). Interestingly, the neo11/435.D1 hybrid cytogenetically did not appear to have an extra chromosome 11, but amplification of dinucleotide repeat regions (Fig. 1*B*) demonstrated that the chromosome 11 from the donor cell line was present in these cells. Because PCR amplification is more sensitive than cytogenetics in detecting the presence of an introduced chromosome, it is possible that the neo11/435.D1 hybrid cells have a subpopulation with the introduced chromosome and the majority of the cells without the chromosome. In contrast, the neo11/435.A3 hybrid karyotypically had an extra chromosome 11, although dinucleotide repeat analysis (Fig. 1*B*) and FISH painting (data not shown) verified that the endogenous chromosome t(11q;13q) was missing. Thus, as verified by karyotype, PCR, and FISH, the microcell hybrids contain a neo-tagged chromosome 11 introduced from the MCH556.1c5 donor cell line. *In vitro* growth rates of the microcell hybrids were not significantly different from that of the

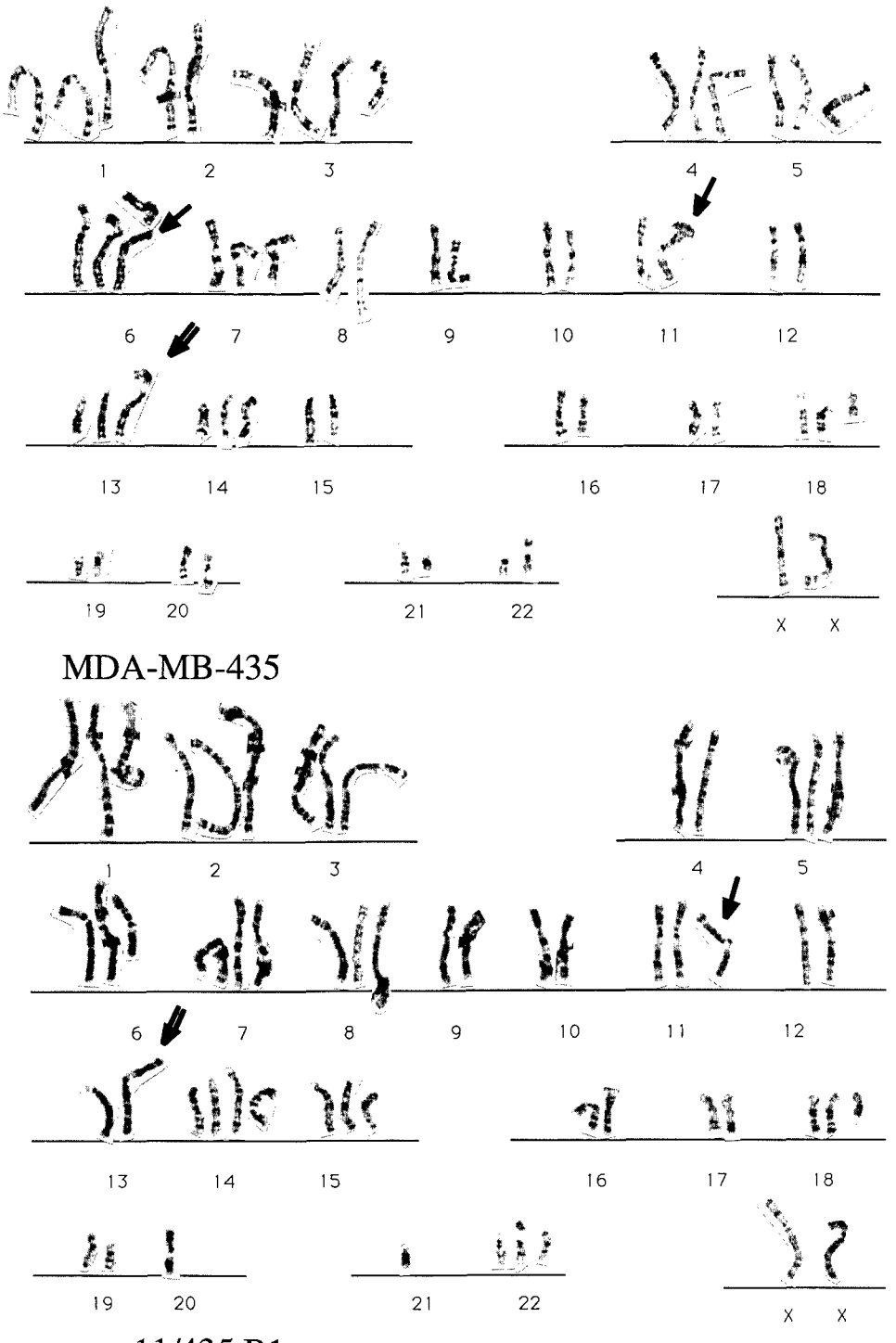

Fig. 2. Representative karyotypes of MDA-MB-435 and neo11/435.B1. *Single arrows,* the derivative chromosomes 6 and 11. *Double arrow,* chromosome t(11q;13q).

MDA-MB-435 recipient cell line, except that neo11/435.E1 cells were larger and had a 2-fold increase in population doubling time (data not shown).

Microcell hybrids containing an introduced chromosome 6 were generated by fusion of a subclone of MDA-MB-435 (MDA-MB-435.7) with the MCH262A1.D6 donor cell line. Several attempts to fuse chromosome 6 microcells from the donor cell line to the MDA-MB-435 mass population were unsuccessful. It was thought that this problem was the result of a gene dosage effect, since the mass population had from two to four copies of chromosome 6 among different cells. Therefore, MDA-MB-435 was subcloned in an attempt to identify colonies with fewer copies of chromosome 6. Cytogenetic examination revealed that subclone 7 (MDA-MB-435.7) had one normal chromosome 6, one derivative chromosome 6, and a marker chromosome containing a fragment of chromosome 6 [t(6;?)]. This subclone easily fused with chromosome 6 microcells from MCH262A1.D6, resulting in four microcell hybrids designated neo6/435.7.A1, neo6/435.7.C1, neo6/435.7.E2, and neo6/435.7.G1. The hybrids were expanded in G418-containing media. Cytogenetic analysis showed that the four microcell hybrids had two normal copies of

chromosome 6, a derivative chromosome 6, and the t(6;?) marker chromosome. Amplification of polymorphic sequences verified that the extra chromosome 6 was introduced into all four microcell hybrids from the donor cell line (Fig. 1). One of the microcell hybrids, neo6/435.7.G1, was largely polyploid (>50%); however, a copy of the extra chromosome 6 was present in all of the cells. *In vitro* growth rates of the microcell hybrids did not differ significantly from that of MDA-MB-435.7 (data not shown).

Tumorigenicity and Metastasis Assays. To determine the potential effects of chromosome 11 on the tumorigenic and metastatic abilities of the MDA-MB-435 cell line, the chromosome 11 microcell hybrid cells were inoculated into the mammary fat pads of athymic nude mice. Table 1 lists the tumorigenic and metastatic properties of these cell lines. MDA-MB-435 and all four of the chromosome 11 microcell hybrids formed tumors following injection, indicating that chromosome 11 does not encode a dominant tumor-suppressor gene for this tumor type. However, the incidence of lung metastases (macroscopic and microscopic) from the four neo11/435 microcell hybrids was significantly different from that of the metastatic parent. MDA-MB-435 cells metastasized to the lung in 93% of the mice, with a mean number of 20 ± 6 (± SEM) metastases per animal. In contrast, all four of the chromosome 11 microcell hybrids had a reduced incidence of metastases in the lung and extrapulmonary sites, as well as a mean number of less than two lung metastases per animal. Metastasis suppression in neo11/435.B1 was most significant ($P < 0.001$), despite a faster population doubling time than MDA-MB-435 (data not shown). Only 6 mice of 29 had 1–2 metastases with neo11/435.B1, even if the primary tumor was surgically removed, allowing time for subclinical microscopic metastases to become visible. neo11/435.A3 had an low incidence also (9 mice of 24); however, one of those 9 mice had 39 metastases with all of the others having 0–5 metastases. Therefore, the mean number of metastases was less than one for neo11/435.B1 and greater than one for neo11/435.A3. All experiments were repeated at least twice, except for neo11/435.E1, because this hybrid was so difficult to grow *in vitro*. Interestingly, the *in vivo* growth rate for neo11/435.E1 was indistinguishable from MDA-MB-435 cells. Perhaps the smaller number of inoculations could explain the less significant $P < 0.05$ for this hybrid. All four neo11/435 hybrids were suppressed for lung metastases by at least 95%. Likewise, the incidence of development of regional lymph node metastases was reduced from 75–100% in MDA-MB-435 and MDA-MB-435.7 to less than 20% for the neo11/435 hybrid cell lines. The RMP, which normalizes the number of metastases in mice injected with the microcell hybrid clone to that in mice injected with MDA-MB-435, also indicates metastasis suppression in the hybrid clones. Three of the four neo11/435 clones had a significantly reduced RMP (Table 1). This RMP difference was less significant in the fourth clone, neo11/435.E1, again probably due to the slower growth and fewer number of inoculations with this clone. These results indicate that the presence of chromosome 11 suppresses the metastatic ability of the MDA-MB-435 breast carcinoma cell line.

Similar tumorigenic and metastatic analyses were performed on MDA-MB-435.7 (the recipient line for chromosome 6 fusions) and the neo6/435.7 microcell hybrids. MDA-MB-435.7 formed tumors and metastases in 100% of the inoculated mice. However, the mean number of lung metastases was only 7, indicating that this subclone was less aggressive metastatically than the mass population (RMP, 0.35). None of the chromosome 6 microcell hybrids was suppressed for tumorigenicity. Only one of the four chromosome 6 microcell hybrids, neo6/435.7.G1, had a marked reduced incidence of metastases (6 of 20); however, this difference was not statistically significant from MDA-MB-435.7. These data suggest that chromosome 6 does not suppress metastasis in human breast carcinoma.

Previous reports determined that nm23-H1 (NME1) functions as a metastasis suppressor protein in MDA-MB-435 (27). To determine whether metastasis suppression in neo11/435 hybrid clones was associated with up-regulation of nm23-H1 (encoded on chromosome 17), Northern blot analyses were performed. Equivalent amounts of *nm23-H1* transcripts were observed in the metastatic MDA-MB-435 line and in the suppressed neo11/435 hybrids (Fig. 3). Likewise, expression of the proteins ER (coded on chromosome 6) and PR (encoded on chromosome 11) were unaffected by introduction of microcells (data not shown).

Table 1 *Tumorigenicity and metastatic analysis of microcell hybrids*

		Metastasis from mammary fat pad			
		Lung			Other sites[b]
Cell line	Tumorigenicity[a]	Incidence[c]	Mean ± SEM	RMP[d]	Incidence (%)[c]
MDA-MB-435	27/27	25/27	20 ± 6	1.00	>75
neo11/435.A3	24/24	9/24	1 ± 0.6	0.05[e]	10–20
neo11/435.B1	29/29	6/29	<1	<0.05[e]	<10
neo11/435.D1	24/24	5/24	1 ± 0.31	0.05[e]	<10
neo11/435.E1	12/12	4/12	1 ± 0.47	0.05[e]	<10
MDA-MB-435.1	11/11	11/11	33 ± 17	1.69	>75
MDA-MB-435.7	7/7	7/7	7 ± 3	0.35	>75
neo6/435.7.A1	16/16	13/16	3 ± 2	0.43[f]	25–50
neo6/435.7.C1	8/8	7/8	5 ± 1	0.71[f]	25–50
neo6/435.7.E2	8/8	8/8	10 ± 4	1.43[f]	25–50
neo6/435.7.G1	20/20	6/20	<1	<0.14[f]	<10

[a] Tumors reached 1 cm in size at 45–55 days postinjection for the recipient cell lines and the neo6/435 clones. Most neo11/435 clones reached 1 cm in <45 days.

[b] Metastases found in adrenals, regional lymph nodes, rib cage, and brain. Percentage of mice with extrapulmonary metastases/number of mice injected is shown. The numbers are normalized for concurrently performed experiments. Highly metastatic lines developed metastases in a greater percentage if tumors remained *in situ* longer.

[c] Number of mice with metastases/number of mice injected or percentage (pooled from 1–3 experiments).

[d] Ratio of metastases compared to parental MDA-MB-435. For neo6/435.7 hybrids, ratio is compared to MDA-MB-435.7.

[e] Statistically significant results using one-way ANOVA with pairwise analysis and Tukey's Honestly Significant Difference test: $P < 0.05$ for neo11/435.E1; $P < 0.01$ for neo11/435.A3 and neo11/435.D1; and $P < 0.001$ for neo11/435.B1.

[f] Not significantly different from MDA-MB-435.7.

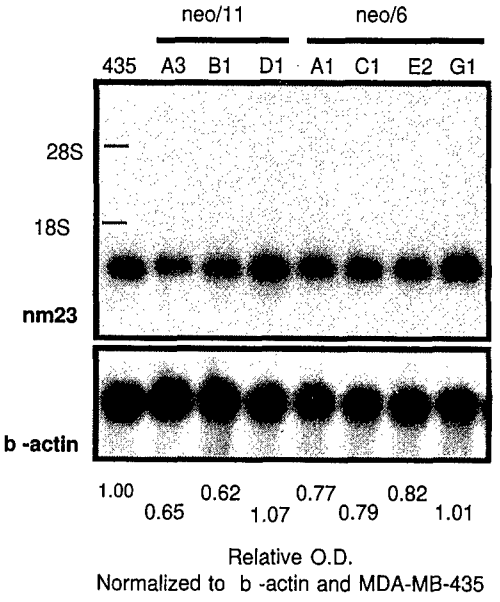

Fig. 3. Northern blot analysis of the MDA-MB-435 recipient cell line, three neo11/435 clones, and four neo6/435.7 clones, probing with the 0.8-kb *Bam*HI *nm23-H1* fragment. Poly(A)$^+$ mRNA (2.5 μg) was loaded into each lane. The same blot was stripped and reprobed with β-actin as a loading control.

Discussion

The purpose of this study was 2-fold: (*a*) we wanted to determine the role of chromosome 11 in breast cancer progression, since a high frequency of chromosome 11 alterations has been detected in late-stage breast carcinoma (7, 28); and (*b*) because our previous studies demonstrated that introduction of a normal human chromosome 6 suppresses the metastatic phenotype of the C8161 melanoma cell line (4) and other human melanoma cell lines,[5] we wished to determine if a common metastasis-suppressor gene for both breast cancer and melanoma existed. To address these questions, neo-tagged human chromosomes 6 and 11 were introduced into MDA-MB-435 human breast carcinoma cells. All microcell hybrids remained tumorigenic in nude mice. However, four neo11/435 clones were significantly suppressed for metastasis to lungs and regional lymph nodes. None of four neo6/435.7 clones was reduced for metastasis when compared against the MDA-MB-435.7 recipient cell line. Taken together, these results suggest that chromosome 11, but not chromosome 6, encodes a breast cancer metastasis-suppressor gene.

Cutaneous malignant melanoma and breast cancer are distinct types of cancer involving different types of cells; therefore, it may seem unlikely that a single suppressor gene could be important in both malignancies. However, there are proteins that could be utilized by both cell types, such as surface adhesion molecules and proteinases. Since loss of cell surface molecules has been shown to correlate with metastatic progression (29), it is possible that loss of a single gene on chromosome 6 or chromosome 11 could result in acquisition of metastatic ability for both breast and melanoma tumor types. Furthermore, LOH data support the involvement of chromosome 6 alterations in both tumor types (4, 7), although these alterations are generally late events only for melanoma. Therefore, it seemed reasonable to test whether the melanoma metastasis-suppressor gene on chromosome 6 might function in breast cancer. Our data indicate that chromosome 6 does not suppress breast cancer metastasis, although definitive evidence awaits further testing. If correct, this would suggest that other genes may act in a tissue-specific manner to suppress metastasis.

[5] D. R. Welch and B. E. Weissman, unpublished observations.

A previous study has indicated that transfection of *nm23-H1* (NME1 protein) into MDA-MB-435 reduced metastasis while not affecting tumorigenicity (27). Although NME1 protein is encoded on human chromosome 17, it is possible that gene expression from either chromosome 11 or chromosome 6 could *trans*-activate, resulting in increased NME1 expression and suppression of metastasis. However, our results indicated there was no difference in the mRNA levels from this gene when comparing chromosomes 6 and 11 microcell hybrids. Therefore, it is likely that a novel gene on chromosome 11 is directly responsible for the metastasis suppression in the neo11/435 hybrids.

Recently, chromosome 11 was implicated in the progression of rat prostate tumor cells to metastasis (30). The *kai-1* gene on human chromosome 11p11.2 was shown to suppress metastasis when transfected into rat prostate cancer cells, yet there was no suppression when chromosome 11 was introduced into rat mammary cancer cells via MMCT. Preliminary data by Western blot analysis indicate that Kai-1 protein expression appears higher in neo11/435 hybrids than in MDA-MB-435, MDA-MB-435.7, and neo6/435 hybrids (data not shown). However, the levels of Kai-1 vary over a wide range in the recipient cell lines. Therefore, the true test for the potential role of Kai-1 in controlling metastasis in these cells will come from gene transfection experiments.

Interpretation of the chromosome 6 hybridization experiments was complicated due to the less aggressive nature of the MDA-MB-435.7 recipient. The fact that three of the four neo6/435.7 subclones were similarly metastatic argues that chromosome 6 does not encode a breast cancer metastasis-suppressor gene. The very low metastatic potential of neo6/435.7.G1 is most likely explained by clonal variation induced by isolation of single-cell clones from mass heterogeneous populations of cells (as was done to derive MDA-MB-435.7; Ref. 31). An additional subclone of the mass population, MDA-MB-435.1, was as metastatic as the unsubcloned parent (Table 1), and chromosome 6 has been successfully introduced to these cells (designated as neo6/435.1 hybrids). Additional experiments are under way to determine the metastatic potential of neo6/435.1 hybrids and to prepare neo11/435.1 and neo11/435.7 hybrids so that we can better determine the impact of these chromosomes on metastasis.

In summary, our results present the first functional evidence for altered gene expression on chromosome 11 contributing to human breast cancer metastasis. Our results highlight the distinctiveness of the genetics controlling the tumorigenic and metastatic phenotypes. Moreover, the results presented here demonstrate that unique metastasis-suppressor genes are operative in tumors of different cellular origin. This cell system should prove a valuable resource for the identification of the operative metastasis-suppressor genes.

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ISOLATION AND INITIAL CHARACTERIZATION OF THE HUMAN METASTASIS-SUPPRESSOR GENE *KiSS-1*

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Using a modified subtractive hybridization technique to compare metastatic human melanoma cells to nonmetastatic chromosome 6-melanoma hybrid clones, we isolated seven different cDNA clones exhibiting quantitatively or qualitatively higher expression in neo6/melanoma hybrids. Of these isolates, a novel cDNA, designated *KiSS-1*, was particularly interesting. *KiSS-1* expression was detectable in cell lines derived from normal melanocytes and nonmetastatic radial growth phase melanoma cells but not in vertical growth phase melanomas or metastatic melanomas, suggesting that *KiSS-1* expression might correlate inversely with progression toward increasing malignancy. Transfection of full-length *KiSS-1* cDNA into five human melanoma cell lines (C8161, MelJuSo, A375M, M24met and C8161cl.9) resulted in significant (50-95%) suppression of metastasis following intradermal and/or intravenous injection into athymic nude mice. Tumorigenicity was not affected. *KiSS-1* transfectants were unaffected for invasion and adhesion to endothelial monolayers or immobilized extracellular matrix components. Surprisingly, the *KiSS-1* gene maps to chromosome 1q32-q41, implying that the acquisition of metastatic potential in these melanoma cells was the result of defective regulation of *KiSS-1* expression rather than mutation of the gene.

Chromosome 1q aberrations are infrequent in most cancers, but occur in some late-stage human breast carcinomas. To test whether *KiSS-1* is a functional metastasis-suppressor in breast carcinoma, parental, vector-only transfectants and *KiSS-1* transfectant clones of the human breast carcinoma cell line MDA-MB-435 were injected into the mammary fat pads of athymic nude mice and assessed for tumor growth and metastasis to regional lymph nodes and lungs. *KiSS-1* expression reduced metastasis by 95% compared to control cells but, as above, did not suppress tumorigenicity. Metastasis suppression correlated with a decreased clonogenicity in soft (0.3%) and hard (0.9%) agar. While the overall rate of cell adhesion to extracellular matrix components was unaffected, *KiSS-1* transfectants spread more rapidly on immobilized type-IV collagen than control populations.

The *KiSS-1* cDNA predicts a predominantly hydrophilic, 164 a.a. protein with a proline rich domain indicative of an SH3 ligand (PXXP motif) and a putative protein kinase C-α phosphorylation site (SMR). Based upon this predicted structure, our collective results imply a mechanism whereby KiSS-1 regulates events downstream of cell-matrix adhesion, perhaps involving cytoskeletal reorganization. This pathway appears to be important for both breast and melanoma tumor cell metastasis.

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<INIT>SF
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<ABSTRACT>KiSS-1 was identified as a melanoma metastasis-suppressor gene using subtractive hybridization between a metastatic human melanoma cell line C8161 and nonmetastatic chromosome 6-C8161 microcell hybrids. The KiSS-1 gene maps to chromosome 1q32-41. Since 1q deletions are often observed during breast cancer progression, we tested whether KiSS-1 would also suppress metastatic potential of human breast cancer cells. The metastatic MDA-MB-435 cell line, in which KiSS-1 transcript is not detectable, was transfected with a constitutive KiSS-1 expression vector. Single cell clones were isolated and metastasis from the subaxillary mammary fat pads was assessed in 5 week-old, female athymic nude mice. Lung and regional lymph node metastasis of KiSS-1 transfectants was significantly ($P<0.05$) suppressed compared to parental cells. Tumor growth *in vivo* and cell growth *in vitro* were slower in KiSS-1 transfectants. Taken together, these results suggest that the KiSS-1 gene might be involved in progression of human breast cancer cells toward malignancy. Furthermore, these results imply that KiSS-1 may be a gene(s) encoded on chromosome 1 in a region previously implicated in breast cancer progression by loss of heterozygosity studies. Supported by U.S. Army grant DAMD-17-96-1-6152, PHS grant CA62168 and the National Foundation for Cancer Research.

DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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